

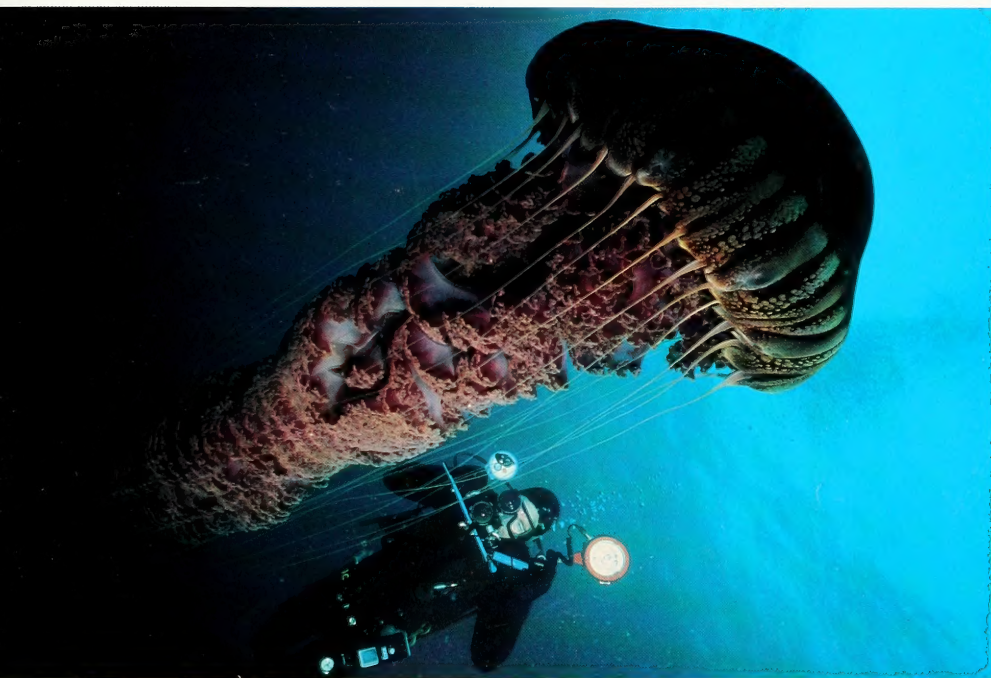


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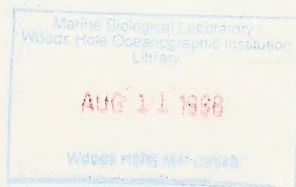
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THE BIOLOGICAL BULLETIN



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THE BIOLOGICAL BULLETIN

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Cover

Chrysaora achlyos, a new, giant species of scyphozoan jellyfish from the eastern Pacific, is described in this issue (see Martin *et al.*). The species name—an artificial construct based on the Greek word *achlys*, meaning mist, darkness, or obscurity—was chosen to reflect both the color of the bell and the fact that the animal has been observed so rarely that we know little about its natural history. Worth noting, however, is that one of its close (though much smaller) relatives is the infamous stinging nettle (*C. quinquecirrha*) found in Chesapeake Bay and elsewhere in the western Atlantic. Photo courtesy of Howard Hall Productions; used with permission.

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The Biological Bulletin accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

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2. **Title page.** The title page consists of a condensed title or running head of no more than 35 letters and spaces, the manuscript title, authors' names and appropriate addresses, and footnotes listing present addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations.

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A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g.*, *J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. All abbreviated components must be followed by a period, whole word components must *not* (*e.g.*, *J. Cancer Res.*)

D. Space between all components (*e.g.*, *J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafélags Íslendinga* without abbreviation.

F. All single word journal titles in full (*e.g.*, *Veliger*, *Ecology*, *Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e.*, *Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g.*, *Nature*, *Science*, *Evolution* NOT *Nature*, *Lond.*, *Science*, *N.Y.*; *Evolution*, *Lancaster*, *Pa.*)

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Charles Baker Metz (1916-1997)



Charles B. Metz was born in New York City on December 27, 1916, the first child of Charles William Metz and Blanche Stafford Metz. His father, a professor of genetics, spent summers at the Marine Biological Laboratory and brought the family along to their cottage on Hyatt Road in Woods Hole, Massachusetts. Thus began young Charles' love for the sea and its creatures and his passion for fishing and boating. Among Charles' early remembrances, working visits to his grandfather's ranch near Sheridan, Wyoming, were favorites and led to an active interest in Native American culture and its artifacts.

Metz's boyhood experiences forged a fiercely independent man of uncompromising integrity. His interests in science and nature having been encouraged by his father, he became an enthusiastic and venturesome experimental biologist. Moreover, his early association

with the MBL continued throughout his life. He was an F. R. Lillie Fellow and an instructor in the embryology course; he developed and ran a renowned training program in fertilization and gamete physiology, was an active member and trustee of the M.B.L. Corporation, and was the ninth editor of *The Biological Bulletin*.

Having received his bachelor's degree from Johns Hopkins University in 1939, Metz went west to do his doctoral work in the laboratory of Albert Tyler at the California Institute of Technology. His dissertation research, completed in 1942, concerned the biochemical interactions between sperm and eggs, and this remained the central theme of his work throughout his 45-year career. He studied the complex details of fertilization in a wide variety of species, from marine invertebrates to mammals. And he also investigated the analogous process of cell-cell recognition that precedes the mating of ciliated protozoans.

It was, particularly, the parallels between fertilization, immunity, and ciliate mating that continued to intrigue Metz—these processes all involve a biochemical recognition between complementary proteins on the surface of interacting cells. Thus he was led to use a variety of specific protein inhibitors, including antibodies and lectins, in studies of fertilization. Armed with this molecular toolbox, Metz probed the functions of various components of sperm and eggs. Years of painstaking laboratory research with his colleagues produced a wide variety of information about the ordering and biochemical features of protein interactions during fertilization. This work contributed substantially to our current understanding of fertilization as an intricate series of molecular recognition events between sperm and egg. Metz's research also influenced and contributed to current studies of the pharmacological, medical, and sociological aspects of the biochemistry of fertilization in humans, including studies of infertility, *in vitro* fertilization, and contraception.

In collaboration with his students and associates,

Metz published more than 120 research papers. He and his colleague Alberto Monroy co-edited two major compilations of fertilization research, which were among the best known and widely cited works in the field: *Fertilization* (two volumes), published in 1967–1969, and *Biology of Fertilization* (three volumes), published in 1985.

His formal education completed, Metz served briefly on the faculties of Wesleyan University, Yale, the University of California at Berkeley, and the University of North Carolina before settling into the Department of Zoology at the Florida State University. In 1953, when Metz arrived, F.S.U. was in the midst of a postwar expansion exacerbated by a conversion from its former life as the Florida State College for Women. To cope with the need for housing and offices, a vacated military base had been turned over to the University, and one of the former wooden barracks on this base was given to Metz for his laboratory. The building was primitive; but it was spacious, the site was lovely, and most important, it was about three miles from the main campus and equally far, therefore, from the administrative reach of the Zoology department. And that suited Metz perfectly.

In 1955, Sydney W. Fox came to F.S.U. to renovate and direct the Oceanographic Institute. Metz became Associate Director of the institute and, as a consistent user of its marine laboratory at Alligator Harbor, directed that facility from 1958 to 1962. In 1961, Metz and Fox established, with the support of NASA, the Institute for Space Biosciences. Both then left Florida State in 1964 to co-found the Institute of Molecular and Cellular Evolution at the University of Miami. Metz remained a Professor of Zoology at Miami until his retirement in 1985.

Research training was one of Metz's major interests; he advised 11 doctoral students and supervised 15 postdoctoral researchers at Florida State and the University of Miami. His first doctoral student, Richard S. Young, had accompanied him from North Carolina to F.S.U. Much later, Dick Young became a valued colleague. While at the Army Ballistic Missile Agency in Huntsville, Alabama, in charge of the first biological experiments to be flown in space (in the recoverable nose cones of Redstone rockets), Dick enlisted Metz's collaboration in testing the notion that microgravity would affect the cleavage of sea urchin eggs.

Late in the 1950s, while still at F.S.U., Metz became convinced that graduate students should have the opportunity, in an informal setting, to present their research and discuss it with experienced investigators. He therefore organized and convened (at the Alligator Harbor marine laboratory) the first Southeastern Develop-

mental Biology meeting. These meetings still continue. Moreover, people who attended the early events were inspired to become organizers themselves; thus, similar conferences in other regions and other fields were propagated. Metz and his graduate students and postdocs were regulars; each year they would load into station wagons and go off to the regional meeting—not failing, of course, to enjoy side trips to places of natural or historical interest.

The Marine Biological Laboratory was the site of Metz's most important and long-lasting accomplishment: the development of an intensive research training program in Fertilization and Gamete Physiology (FERGAP). This program was supported by the National Institutes of Health and was taught for many years by Metz, C. R. Austin, Alberto Monroy, and Leonard Nelson. With time, others signed on, and the staff came to have a distinctly international flavor. For 13 years (1962–1974), FERGAP provided graduate students, postdoctoral researchers, and professors with a unique opportunity to work together and to participate in advancing the field of fertilization. The varied research interests of the staff, and the availability of pertinent equipment, enhanced the opportunity to develop research projects in morphology, immunology, or molecular biology. Over the years, the trainees in the program included many who are still active today in fertilization and associated areas.

In 1979, Metz took over the editorship of *The Biological Bulletin* from W. D. Russell-Hunter and ran the journal for 10 years. Under his supervision, the appearance of the Bulletin changed markedly, and for the better. The “classic” grey cover with its black lettering was brightened, and the page size was increased; the format was changed to two columns, so it became easier to read; and the quality of the photoreproduction was increased to its present high standard. To accompany the face-lift, Metz began to shift the focus of the journal back toward experimental biology—particularly in the areas of development and reproduction—and he laid out a series of regular reviews from prominent biologists.

Metz spent virtually every summer at Woods Hole. As the season ended, he would make a last, large extract of sea urchin egg-jelly (fertilizin), pour it into lengths of dialysis tubing, suspend the bags from the roof inside his station wagon, and then start driving to Florida. Water would slowly ooze out of the bags, but the flow of air through the wagon would evaporate it, cooling the extract while concentrating it. By the time he reached Tallahassee, the fertilizin titre of the extract was quite high. Of course, the whole trick depended on the station wagon being driven very fast and with only necessary stops—never a problem for Metz.

Charles Metz was married for 44 years to Ethel Buckheimer who passed away in 1984 after a long illness. They had two children, Rinda and Richard. In 1985, he married Grace S. Metz, and they spent the next decade living in Miami and Woods Hole, and sailing intrepidly (through three hurricanes and the loss of their rudder) between the two spots.

Metz *always* had a boat. He would use his boat for fishing, for collecting, for transport to picnic sites with students, for sightseeing trips with visiting dignitaries, to test his powers of navigation, for the pure pleasure of being out on the water, and (in the exigencies) as an anodyne. In the early days, he had a power boat, and he and Ethel would trailer it between Woods Hole and Tallahassee or Miami. In the late seventies and eighties, he turned to sailing, first on the *Brisa*, and then on the *Luidia* (which was named after one of his favorite genera of starfish; his dinghy was the *Bipinnaria*). As in all things that mattered to him, sailing was "serious busi-

ness," and he paid attention to the details. Thus, since it was always critical to conserve space, he only kept 151 proof rum on board.

Charles B. Metz passed away on Tuesday, January 14, 1997, in Homestead, Florida. He had just entered his 80th year.

Bill Eckberg recalls: "In the end, it was a massive stroke that took him from us. The last time we visited him, his illness had taken away much of his ability to communicate, but he still had the old gleam in his eyes when he looked at a painting of a wave he had in his living room, and we could tell he was thinking about sailing. Once a few years before, he and Grace were on the *Luidia* following our boat to Nantucket; and suddenly they turned into the wind, dead in the water with all sails luffing. Thinking that they must be in trouble, we turned back anxiously—only to discover that they'd stopped because they'd been trolling and caught a bluefish! Metz always had his priorities straight."

Gertrude W. Hinsch
William R. Eckberg
Edward Metz
Michael J. Greenberg

Mucociliary Transport in Living Tissue: The Two-Layer Model Confirmed in The Mussel *Mytilus edulis* L.

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The present study combined video confocal laser microscopy (1) and tissue reflectance and autofluorescence to visualize mucus position and mucociliary transport in excised living gill tissue from the blue mussel Mytilus edulis. Rafts of mucus and embedded particles were transported atop a periciliary space traversed by frontal cilia, which engaged the mucus layer and moved it during the effective stroke, disengaging and completing the cycle during the recovery stroke. These results confirm the two-layer model for mucociliary transport in the mussel gill. Given the conservative nature of ciliated epithelial structure and function (2, 3), and the structural similarity of mucociliary surfaces as diverse as terrestrial vertebrate respiratory epithelium and molluscan gill, the two-layer mechanism of mucociliary transport may be a general feature of Metazoan biology.

Mucociliary transport is a common mechanism of particle movement along epithelial surfaces in many Metazoan taxa (4, 5, 6). Examples include cleaning of respiratory surfaces in both terrestrial vertebrates and aquatic invertebrates, as well as suspension-feeding in aquatic invertebrates. Indeed, transport of material on terrestrial vertebrate epithelia appears to be impossible without the intervention of mucus as a mechanical coupler (7, 8). In humans, disorders of mucociliary transport are manifested in diseases such as cystic fibrosis and chronic bronchitis (9). The mucus layer also reduces dehydration of respiratory surfaces in terrestrial animals—a prerequisite for life on land. Although the observational techniques employed to date have not yielded con-

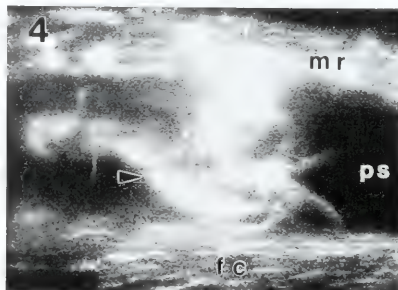
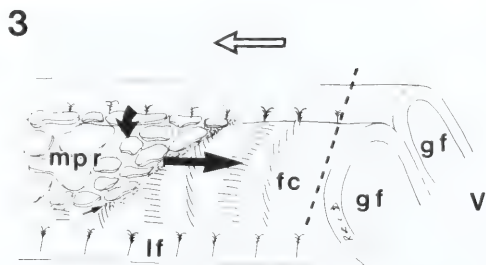
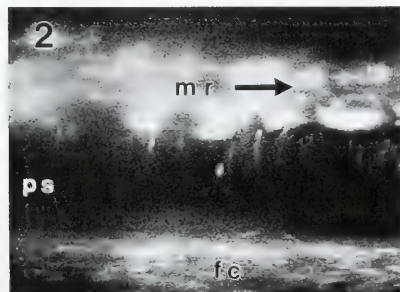
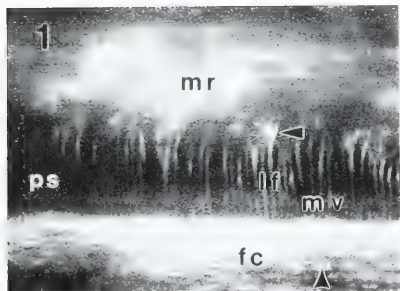
sistent results, some indirect data support a two-layer model, with a viscous mucus layer atop a less viscous periciliary fluid, within which the cilia beat (4, 8, 10, 11, 12, 13). However, the exact position of mucus and mechanism of mucociliary transport have never been observed directly in living tissue. Previous attempts to directly observe mucociliary transport have been confounded by the transparency of mucus and the inability to achieve sufficient field depth to visualize both cilia and three-dimensional transport simultaneously. In the present study, 1- μ m fluorescent particles were used to help render mucus visible after its incorporation in the mucus raft, and the confocal laser technique was employed to visualize fluorescence and autofluorescence with high resolution and achieve sufficient and selectable field depth in live preparations.

Excised bivalve gill tissue is an ideal preparation for the microscopic observation of mucociliary transport, since it is normally immersed in an aqueous medium and ciliary activity remains intact for prolonged periods (14). Pieces of the posterior extremity of each of the four demibranchs of adult *Mytilus edulis* were excised and observed using a modified video confocal laser technique (1); these conditions enabled the visualization of mucus, direction of water flow, and particle transport.

Mucus on the mussel gill was located atop the frontal cilia of each filament, between the apposing rows of latero-frontal cirri (Figs. 1–4). The spatial configuration of the mucus was in the form of rafts that varied from 10 to 25 μ m in thickness, tapering to occasional slight gaps. Such a large range of thickness could be due to unequal secretion, unequal distribution on the surface, or both, as well as to the differential states of hydration of mucus secreted at slightly different times. The physiological stress of sectioning and mounting probably also in-

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Figures 1–2. Lateral view of frontal surface of *Mytilus edulis* gill filament. The two-layer structure is evident, comprising the periciliary space (ps), within which the frontal cilia (fc) beat, and a mucus raft (mr), propelled by the frontal cilia (arrow). The latero-frontal cirri (lf) are visible when within the plane of focus. The played cirral 'V' (upper arrowhead) belongs to one of the shorter cirri, which does not extend over the raft surface. The V width, nuclei (bottom arrowhead) and the height of the microvillar layer (mv) were used for scale calibration.

Figure 3. Interpretive drawing (not to scale) showing two transversally-sectioned gill filaments (gf), latero-frontal cirri (lf), frontal cilia (fc), position and direction of movement of mucus raft with included particle masses (mr, straight solid arrow), direction of latero-frontal cirral beat (curved solid arrow), direction of pallial fluid flow over filaments (hollow arrow), and direction of frontal cilia effective stroke (small arrow). Dotted line represents plane of view in Figs. 1, 2, 4. V, ventral orientation.

Figure 4. Live crustacean larva (arrowhead) trapped within the periciliary space (ps), between frontal cilia (fc) and mucus raft (mr).

Scale bar valid for Figs. 1, 2, 4.

creased the secretion of mucus and thus the thickness of the rafts, and the highly reflective particles may have presented a halo effect that exaggerated the raft thickness. The mucus rafts were transported in the normal direction by the frontal cilia, *i.e.*, toward the ventral margin of the gill (Figs. 1, 2); although observations of their entrainment into the ventral groove were not made, this is the only available destination. It was possible to observe beating of individual frontal cilia only at a recording speed ≥ 120 frames/s, and even under these conditions the uniform contrast presented by the many surrounding cilia precluded detailed observations of individual ciliary beat or clear still micrographs. Video

sequences showed that the tips of individual cilia interacted with the mucus rafts during the effective stroke and disengaged and completed the cycle below the rafts during the recovery stroke. Both the frontal cilia length (about 19–24 μm) and the periciliary space (13.3–15.3 μm) were considerably larger than the corresponding cilia length and putative periciliary space (4–6 μm) in the terrestrial vertebrate respiratory epithelium (4,13); this may be a consequence of the presence of water on both sides of the raft in the aquatic medium of the mussel gill. The frontal cilia length is in line with or only slightly larger than that suggested by previous data for *M. edulis* (15), when curvature, fixation shrinkage, and the height

of the microvillar layer are accounted for. In the present study, scanning electron microscopy of fixed filaments corrected for shrinkage, confocal laser microscopy, and light microscope measurements on live desquamated frontal cells gave good agreement for frontal cilia lengths. The 4–9 μm by which the frontal cilia length exceeded the periciliary space height probably allows both for bending during the effective stroke and for sufficient insertion into the mucus raft.

These observations confirm the two-layer model for mucociliary transport and position on the ciliated epithelium of the mussel gill. The mechanism for mucus positioning above the periciliary space is not yet known, but is probably a combination of capillary action through the periciliary space upon secretion and the subsequent inability of the newly hydrated and cross-linked macromolecules to penetrate between the dense cilia cover (16). The frontal cilia are thus free to beat in the much less viscous (18) periciliary fluid (which is continuous with the water in the palpeal cavity through the gaps in the mucus rafts), and the detailed characteristics of two-layer mucociliary transport (4) are thus consequent. The robustness of the two-layer spatial organization was evident from observations of large pieces of detritus and even an apparently live trapped crustacean larva that presumably entered the periciliary space through a gap in the rafts (Fig. 4).

The observations of the present study show that the latero-frontal cirri beat perpendicular to the mucus raft movement (Fig. 3). Previous reports (1) and work in progress show that these cirri intercept suspended particles and deviate them onto the frontal surface; the present observations suggest that the particles are in fact deviated onto the mucus raft, since most cirral tips extend several micrometers above the raft (deduced from the absence of cirral 'V's at the distal extremities in Fig. 2). This probably represents a major mode of particle capture and initial transport in the mussel—a mechanism that has eluded direct observation to date in any bivalve species.

The role of mucus in bivalve suspension-feeding has been a subject of considerable controversy for decades, focusing on whether it is used in normal feeding or only in the cleaning of feeding surfaces, *i.e.*, the production of pseudofeces (17, 18, 19, 20, 21, 22). Video endoscopy and direct sampling of the contents of the ciliated tracts of bivalves displaying relaxed, normal feeding behavior has clearly demonstrated that mucus is involved in both cleaning and feeding (5, 21). Subsequent mucocyte mapping has revealed that different types of mucus are used in different anatomical contexts, which themselves are directly related to the different aspects of particle processing (23, 24, 25, 26). In addition, the continuum of mucocyte distribution extends well into the esophagus,

beyond the sites of pseudofeces production (27). Recent opposition to this body of data centers on the conjecture that artifactual secretion of mucus might occur in any non-natural situation (22). These objections would preclude virtually all studies of bivalve feeding mechanisms, as well as most other physiological investigations. Similarly, other workers (28) discount *in vitro* studies altogether, even though some aspects of particle processing have been shown to be unaffected by dissection (25). *In vitro* studies and other manipulations impose limitations, as do all investigative techniques, but their utility should not be arbitrarily rejected (29).

The chief morphological and biochemical features of ciliary beat are essentially identical throughout the Metazoa (30), as are the structure and function of mucociliary epithelia (2, 3, 4, 13). It is thus likely that (with the exception of several phyla that lack cilia) the two-layer mechanism of mucociliary transport is a general feature of Metazoan biology. This simple but elegant two-layer system also serves important functions other than particle transport in the Metazoa. The periciliary fluid layer in terrestrial vertebrates, for example, has assumed a critical role in gas exchange through an aqueous medium and as a solvent for molecules detected by olfaction. The two-layer system is crucial to both the functioning and the maintenance of such basic biological processes.

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Chrysaora achlyos, a Remarkable New Species of Scyphozoan from the Eastern Pacific

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Abstract. An enormous new species of scyphozoan jellyfish, *Chrysaora achlyos*, is described from the eastern Pacific. The description is based primarily on color photographs and video footage of living animals and the morphology of four specimens collected in 1989. The natural history, life cycle, and sporadic appearance of the species all are unknown. The species appeared most recently in large numbers in 1989 but has appeared at least twice previously in this century; published photographs (unlabeled or incorrectly identified) appeared in 1926 and 1965. The species is easily distinguished by its size and coloration from other known species in the genus, all of which are considerably smaller. Morphological characters are described, and limited data on nematocyst types are presented. Because of the size of the new species and the known potency of the sting of congeners, we mention briefly the possible consequences of human contact.

Introduction

In late 1989, an undescribed species of scyphozoan jellyfish was seen in large numbers off southern California and Mexico (Martin and Kuck, 1991). The very distinctive medusa was described as being large, with a bell diameter of 20 to 40 cm in preserved specimens but reaching an estimated 1 m in live individuals. Long, delicate tentacles and relatively thick oral arms extended perhaps 6 m below the bell in life. The bell was mostly dark purple to black in coloration (Martin and Kuck, 1991). Consultation with experts in the field confirmed that the spe-

cies belonged in the genus *Chrysaora* but clearly was none of the known species in that genus (R. Larson, pers. comm.; see also Larson, 1990; Larson and Arneson, 1990). Interestingly, the species had been photographed before but not described; Martin and Kuck (1991) attributed photographs appearing in Crowder (1926) and Halstead (1965) to this species. The Crowder photograph was labeled only "black jellyfish" and was published in a popular magazine. The photographs in Halstead (1965), one of which was reprinted in Halstead (1992), deserve special mention. Both photographs were incorrectly identified as *Cyanea capillata*, a species not at all similar to the new *Chrysaora* in aspects other than size, and were attributed to P. Saunders, who worked exclusively in the Pacific. Halstead's label indicating that the photographs were taken off the coast of Florida is therefore obviously an error. Since 1989 the animal has been the subject of several popular articles (see synonymy below) and at least one film (*Nature's Seasons in the Sea*, National Geographic/Thirteen-WNET and Howard Hall Productions, 1990).

Despite the fact that large numbers of individuals came ashore in the fall of 1989, and that shoals of hundreds of these animals were seen and photographed, few collections were made. We are aware of only four specimens. Three are housed in the Natural History Museum of Los Angeles County (LACM), and one is at the Cabrillo Marine Aquarium in San Pedro, California (CMA). All specimens are in poor condition, with only the bell intact. All tentacles are missing, and only pieces of the oral arms remain.

Since the Martin and Kuck paper, concerted efforts have been made to properly describe the new scypho-



Figure 1. *Chrysaora achlyos*, new species, *in situ* photograph taken by members of Howard Hall Productions film crew off Coronado Norte, Islas de Los Coronados, northern Baja California, Mexico, July 1989. Side view of intact animal in front of diver; bell diameter estimated at 1 m. Note distinctive color pattern, length of tentacles, and especially length of combined oral arms (estimated to be 6 m), which trail to bottom right of photograph (terminating beyond frame of photograph). Used by permission of Howard Hall Productions.

zoan on the basis of nematocyst ultrastructure, allozyme electrophoresis, immunology, and morphology. Unfortunately, because of the paucity of specimens and their poor condition, a full description of this interesting animal by modern standards apparently is not possible. We describe it here primarily on the basis of gross morphology, color, and limited information on nematocyst types.

Materials and Methods

Specimens were collected either dead or dying in the surf zone from several areas along the southern California coast in late 1989 (see *Material examined* under Results and also Martin and Kuck, 1991). Photographs of living animals (Figs. 1, 2) and video footage taken off the coast of Coronado Norte, Islas de Los Coronados, northern Baja California, Mexico, in July 1989, were kindly sent to us by Howard Hall of Howard Hall Productions. All three preserved LACM specimens were initially fixed in formalin and later transferred to 70% ethyl alcohol. These specimens, preserved in 1989, were subjected to histological examination 7 years later. Tissue samples were taken from the edges of the bell and lappets, and were embedded in paraffin and stained with hematoxylin and eosin as well as Masson's trichrome. The sole CMA

specimen was preserved in ethyl alcohol and was not subjected to histological examination.

Results

Order Semaestomeae

Family Pelagiidae

Chrysaora achlyos, new species

Black Jellyfish.—Crowder, 1926: 190 (photograph with caption only).

Cyanea capillata (Linnaeus).—Halstead, 1965: plate 43 (misidentification of two P. Saunders photographs, incorrectly attributed to coast of Florida).—Halstead, 1992: plate 86 (one of the same photographs that appeared in Halstead, 1965).

Giant pelagic jellyfish.—Hall, 1990: 29, 2 unnumbered figures.

Purple jellyfish.—Cranston, 1993: 2–3 (photograph with caption only).

Large Jellyfish.—Whiteman, 1996: 19 (photograph by H. Hall).

Unlabeled figure.—Straus and Lisowski, 1997: 509 (photograph by H. Hall).

Chrysaora sp.—Larson, 1990: 549 (Table 1).—Martin and Kuck, 1990: 64 (abstract).—Larson and Arneson, 1990: 130.—Kuck and Martin, 1990: 48 (two photographs by H. Hall).—Martin and Kuck, 1991: 89, figs.



Figure 2. *Chrysaora achlyos*, new species, ventral view of different specimen photographed on same day and in same location as in Figure 1, with diver inspecting oral arms and underside of bell. Note number and arrangement of tentacles and spiraling and interlocking of oral arms. Used by permission of Howard Hall Productions.

1–3, table 1.—Anderson, 1992: 26 (four photographs by H. Hall).—Campbell, 1992: 2, 16, and inside front cover (all photographs by H. Hall).

Material examined: Holotype, CMA Acc. No. 89.28.1 (Cabrillo Marine Aquarium, San Pedro, California), female, bell diameter approximately 22 cm, found dead by staff members of the Cabrillo Marine Aquarium, Los Angeles Breakwater Light, near Long Beach, California, 15 July 1989. Paratypes: LACM 89-206.1 (Natural History Museum of Los Angeles County), probable male, bell diameter approximately 23 cm, found live approximately 100 m offshore of Venice Beach, California, 20 August 1989, collected by B. T. Hogue and D. Golles. LACM 89-205.1, sex undetermined, bell diameter approximately 25 cm, found alive, collected on 25 August 1989 by LACM Ichthyology Section, just north of the Marina del Rey jetty, Venice, California. LACM 89-26.1, sex undetermined, bell diameter approximately

24 cm, found dead floating on surface in the surf zone, Venice Beach, California, collected 25 August 1989 by J. Martin and H. Kuck.

Description

Bell: Hemispherical, fleshy, large; up to 25 cm diameter in preserved specimens examined; reaching estimated 1 m bell diameter in life (based on still photographs, video footage, and eyewitness accounts; see Figs. 1, 2, and photographs in Hall, 1990; Anderson, 1992; Campbell, 1992; Cranston, 1993). Surface of bell smooth, lacking nematocyst warts (see Russell, 1970).

Marginal lappets: 32; all square with rounded corners.

Marginal sense organs: 8; each with two rhopalial cones, one from exumbrella and extending inward, one between and formed by junction of adjacent lappet borders and directed upward. Opening of exumbrellar cone strongly triangular in some specimens (e.g., LACM 89-206.1). Both cones wide and deep; e.g., in specimen LACM 89-205.1 exumbrellar cone approximately 7.8 mm diameter and 5.5–7.0 mm deep; rhopalial (lappet) cone slightly narrower but deeper, 7.2–6.9 mm in diameter and 8.0–9.0 mm deep. Rhopalial interradial and perradial, and set into cones approximately 1 cm from margin of bell.

Tentacles: 24; white to light pink in color (from various still photographs and video footage), each arising between lappets, in sets of 3 between adjacent rhopalial. Each with basal swelling, with one side more protrusive than other (Fig. 2). Long, extending to perhaps $\frac{1}{2}$ to $\frac{3}{4}$ length of oral arms; delicate.

Manubrium: Thick, stiff, partly encircling 4 interradial subumbrellar ostia, through the center of which protrude thick fingerlike projections bearing gonads in mature individuals.

Oral arms: 4; perradial, extremely large, margins frilly, spiraling and interlocking in “corkscrew” manner, twisting in counterclockwise direction as viewed from above bell and extending to some 6 m below manubrium in live animals (Figs. 1, 2).

Radial septa: 16; two between adjacent rhopalial, each terminating on center of lappet.

Gonads: 4, interradial, surrounding and protruding through subumbrellar ostia and borne on ends of fingerlike projections (see manubrium above).

Color: Bell dark purple to black, opaque. Oral arms lighter, more purple than black. Dark red-brown mucus produced when handled (Martin and Kuck, 1991). Perimeter with distinctive light brown to tan spotted or reticulated pattern extending upward to approximately $\frac{1}{5}$ to $\frac{1}{4}$ height of bell (Fig. 1). No variation encountered; all specimens seen or collected exhibited same color pattern. All color faded to a translucent light brown in preserved individuals.

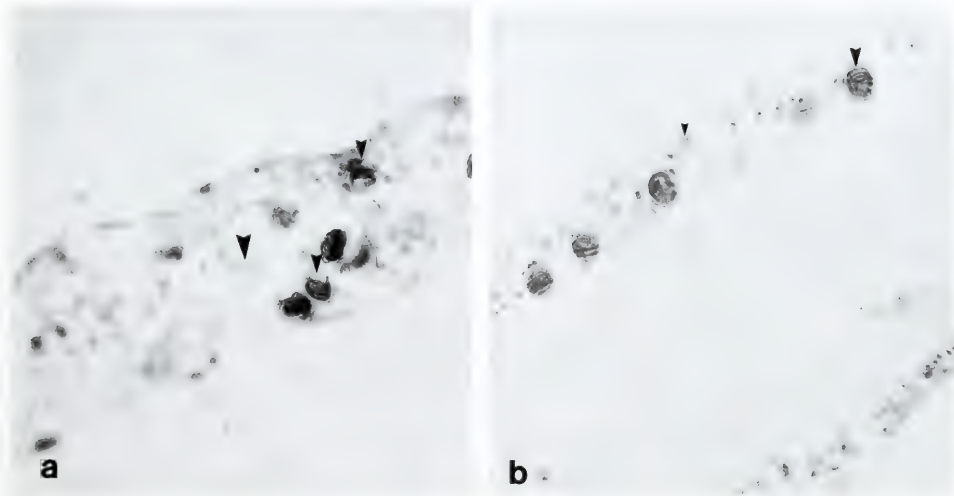


Figure 3. *Chrysaora achlyos*, new species, selected cnidocytes from biopsy of bell perimeter. (a) Section of bell perimeter containing isorhiza (large arrow) and several large euryteles, two of which are indicated (medium-sized arrows). (b) Adjacent section of bell perimeter containing several large euryteles, one of which is indicated (medium-sized arrow), and smaller euryteles (small arrow). Magnification of both photographs is $400\times$.

Nematocysts: Four types in one sample from edge of bell (Fig. 3). Cnidom identical to that of *C. quinquecirrha* from Chesapeake Bay (Burnett *et al.*, 1968; Sutton and Burnett, 1969). A large isorhiza of $15 \times 22 \mu\text{m}$ diameter ($\pm 5\%$, $n = 15$), a smaller round isorhiza with a few coiled threads (rare; size variation not measured), a $14 \times 7 \mu\text{m}$ eurytele ($\pm 14\%$, $n = 30$), and a cluster of smaller, $4 \times 7 \mu\text{m}$ nematocysts ($\pm 8\%$, $n = 15$) resembling the larger euryteles were identified (Fig. 3a, b) (see Discussion for comments on size variation).

Life cycle: Unknown.

Distribution: Isla San Quentin, Baja California, Mexico, north to Santa Monica Beach, California (Martin and Kuck, 1991).

Etymology: An artificial construct based on the Greek *achlys*, meaning mist, darkness, and obscurity (Brown, 1956), referring both to the dark coloration and infrequent appearance of this remarkable species.

Discussion

Behavioral notes, including size of the shoals, known or suspected symbionts, associates, parasites, and possible predators, are found in Martin and Kuck (1991). These authors also established that the first sightings were made along the Baja California peninsula in the summer of 1989 (7 July, 1989, Isla San Martin, San

Quentin, Baja California, Mexico). Subsequent sightings, including those made by the Howard Hall film crew off Coronado Norte, Islas de Los Coronados, Mexico, were to the north, and often involved animals that were moribund (Martin and Kuck, 1991: fig. 1B). The latest sightings were in late August to early September, 1989, and were from La Jolla, California, to Venice Beach and Santa Monica, California, all to the north of Isla San Martin and Coronado Norte, Islas de Los Coronados. Thus, the species appears to have arrived in southern California waters *via* a southern route, coming up along the outer coast of the Baja California peninsula.

Of the five currently recognized species of *Chrysaora* (Arai, 1997), the new species is most similar to *C. plocamia* (see Table I), a species about which very little is known. However, *C. achlyos* can be immediately distinguished by its size and color pattern, which in *C. plocamia* consists of dark radiating stripes upon a translucent bell background. Additionally, a single sentence translated from Haeckel's (1880) description of *C. plocamia* serves to establish a major difference between the two species: "Tentacles as short as the umbrella diameter." In *C. achlyos* the tentacles are perhaps 3 or 4 times longer than the bell diameter (Figs. 1, 2).

Although nematocyst types and sizes are included here, we stress that there is great variability with nematocyst measurements in preserved cnidarian samples, and

Table 1

Comparison of some morphological features and ranges of *C. achlyos*, new species, with the five currently accepted species of *Chrysaora* listed by Arai (1997) and with two species of the closely related scyphozoan genus *Pelagia*, at least one member of which is possibly a member of *Chrysaora* (L.-a. Gershwin, unpubl. data)

Characteristic ¹	Species						
	<i>P. noctiluca</i>	<i>P. colorata</i>	<i>C. achlyos</i>	<i>C. melanaster</i>	<i>C. fuscescens</i>	<i>C. hyoscicella</i>	<i>C. plumbea</i>
Rhopalium pits/cones	pits	cones	cones	cones	cones	cones	cones
Number of tentacles	8	8	24	24	24	24	40
Number of lappets	16	32	32	32-40	32	32	32
Coloration ²	mauve	white with purple stripes	purple/black	brown stripes on pale bell	reddish bell with light streaks	beige/cream with brown chevrons	milky bell with reddish bars
Pattern ³	no star	star	no star	star	star	star	star
Polyp present	no	yes	—	yes	yes	yes	yes
Ephyra nematocysts ⁴	0	1	—	1	1	1	1
Septa	straight	bent	straight	straight	bent	bent	bent
Septa point of termination	off rhopalia	off rhopalia	off rhopalia	—	—	near rhopalia	—
Oral arms	straight	spiral	spiral	straight	straight	straight	straight
Manubrium	elongate	short, heavy	short, heavy	elongate	elongate	elongate	elongate
Bell	small, light	large, heavy	large, heavy	large, heavy	large, heavy	large, heavy	small, light
Maximum size (bell diameter) ⁵	10 cm	90 cm	100 cm	30 cm	30 cm	30 cm	20 cm
Range	Atlantic, Trop. Pacific	S. California	S. California, Baja California Mexico	Bering Sea	Eastern N. Pacific (Oregon, N. California)	British Isles	Peru and Chile
							Eastern N. America

Note: Unknown character states are indicated by a dash (—).

¹ Data from Agassiz and Mayer (1898), Cones (1969), Haackel (1880), Kakinuma (1967), Kramp (1961), Larson (1990), Pages *et al.* (1992), Russell (1970), and personal notes of the second author.

² Refers to ground color of bell and/or the color of the radiating stripes.

³ "Star" indicates a pattern of pigmented stripes radiating from the center of the bell.

⁴ Refers to patterns of nematocyst patches on the ephyra stage, where 0 is the "Pelagia pattern" (e.g., as shown in Russell, 1970: 82, fig. 45) and 1 is the "Chrysaora pattern" (e.g., Russell, 1970: 99, fig. 53).

⁵ Sizes are approximations from the published literature and personal notes, but are in need of verification based on live animals.

that our samples were preserved for 8 years prior to histological examination. Comparison by one of us (JWB) of nematocyst measurements of recent and long-preserved specimens of *Chrysaora quinquecirrha* and also the hydrozoan *Physalia* indicate great variation in measured size due to fixation; sizes given here for *C. achylos* nematocysts probably do not reflect size in life, and are in need of verification with living specimens.

The only person stung by *C. achylos* (J. Martin) recalled a local mild burning sensation that was less intense than stings he received from live specimens of the Chesapeake Bay nettle, *C. quinquecirrha*. However, stings from *C. achylos* were a result of handling the barely living or dead medusae after collecting, and were probably caused by free nematocysts floating in the collecting bucket, as no tentacles remained on any of the collected specimens. *Chrysaora* is generally regarded as a moderately venomous genus of jellyfish. Perhaps the most significant clinical case was that of a victim who developed massive urticaria, lightheadedness, and nausea for many minutes when stung by a host of Chesapeake Bay nettles (*C. quinquecirrha*) falling on him from a broken fishing net hoisted over his head (Hartman *et al.*, 1980, patient A). If such an injury could occur from contact with medusae of smaller nettles (albeit many of them), it might be reasonable to anticipate equally or more severe clinical symptoms in a human that contacted the tentacles of the considerably larger *C. achylos*. Caution is therefore advised in approaching or collecting this species whenever it next appears.

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Chromosome Segregation in Fertilized Eggs From Triploid Pacific Oysters, *Crassostrea gigas* (Thunberg), Following Inhibition of Polar Body 1

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Abstract. Chromosome segregation in fertilized eggs from triploid Pacific oysters, following inhibition of the first polar body (PB1), was studied with acetic orcein staining techniques. To block the release of PB1, fertilized eggs were treated with 0.5 mg/l of cytochalasin B (CB). Four types of segregation were observed, namely, “tripolar segregation” (54.5%), “united bipolar segregation” (12%), “separated bipolar segregation” (2.5%), and “incomplete united bipolar segregation” (4%). The remaining 23% could not be classified because of chromosome disorganization, but appeared to be variants of the above. It seemed clear that the predominant pattern that gave rise to tetraploids was united bipolar segregation, although certain separated bipolar segregations might also lead to the formation of tetraploids. The sequential events of meiosis observed in CB-treated eggs are described. The asynchrony of meiotic events and possible mechanisms for the various types of chromosome segregation are discussed.

Introduction

Viable tetraploids have been produced in the Pacific oyster, *Crassostrea gigas* by blocking the first polar body (PB1) in eggs from triploids fertilized with haploid sperm (Guo and Allen, 1994b). Previous studies of the effect of blocking PB1 on subsequent chromosome segregation in diploid eggs revealed a variety of segregation patterns

during meiosis II, including “tripolar segregation,” “united bipolar segregation,” and “separated bipolar segregation” (Guo, 1991; Guo *et al.*, 1992). It was hypothesized that tetraploids were produced by united bipolar segregation. If this type of segregation occurred in a triploid egg, 30 chromosomes would remain in the egg, and with an additional 10 chromosomes contributed by a haploid sperm, tetraploidy would result (Guo, 1991). However, this hypothesis was not supported by cytological observations in triploid eggs themselves. Only recently have observations on triploid meiosis been made (Guo and Allen, 1994a; Komaru and Wada, 1994). In Pacific oysters, Guo and Allen (1994a) found that the majority of fertilized eggs from triploid female $\times$ diploid male matings (TD crosses) went through two meiotic divisions and released two polar bodies, as did diploid eggs. Survivors consisted of 33% diploids, 5% triploids, and 10% tetraploids. In the tetraploid induction, when TD crosses were treated with cytochalasin B (CB)—so called TDCB crosses—aneuploids (23%), diploids (3%), triploids (3%), and mosaics (3%) were found in addition to tetraploids (67%) at 3 months postfertilization (PF) (Guo and Allen, 1994b). The differences in ploidy composition between TD and TDCB crosses demonstrated that blocking PB1 greatly changed the chromosome segregation in triploid eggs.

The objectives of this study were to reveal the pattern of meiotic segregation in fertilized eggs from triploids following the inhibition of PB1, document the probable cytological explanation for the production of tetraploids, and test the hypothesis that united bipolar segregation can occur in these zygotes.

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Materials and Methods

Triploid Pacific oysters used in this study were 2 years old and produced by blocking the release of the second polar body (PB2) in the fertilized eggs of diploids (Allen *et al.*, 1989). Ploidy was confirmed in all individuals by flow cytometry prior to spawning. Gametes were obtained by strip-spawning. Eggs were passed through a 60- μ m screen to remove the large tissue debris and rinsed on a 25- μ m nytex screen. Sperm was passed through a 25- μ m screen to remove tissue debris. All fertilization and incubation was conducted at 24° to 26°C using filtered (2 μ m) seawater.

Two experimental groups were produced. In the first group, fertilized eggs were allowed to develop as controls, referred to as TD. In the second group, fertilized eggs were treated with 0.5 mg/l CB in 0.5% DMSO, referred to as TDCB. Treatment with CB began at the first indication of PBI extrusion and lasted until about 50% of the developing eggs exhibited PBI in the control. That is, the control was used to gauge the timing of the treatment group that was under the influence of CB. Two replicates were conducted using different pairs of parents.

To study chromosome segregation, samples of developing zygotes were taken every 5 min until 60 min PF and fixed with Carnoy's solution (1:3 glacial acetic acid and absolute methanol). Fixatives were changed twice following light centrifugation. Chromosomes were observed by acetic orcein stain (Guo *et al.*, 1992). Briefly, drops of fixed sample were spread on a slide, mixed with 2–3 drops of orcein stain (0.5% orcein in 60% acetic acid), and covered with a cover glass. After 5 min, the cover glass was pressed gently and sealed with Cytoseal mounting medium. Slides were examined with a Nikon Opiphot microscope. Photographs were taken with Kodak Technical Pan 2415 black-and-white film with speed set at 64 ASA.

Results

Synapsis in eggs from triploid Pacific oyster was characterized by the presence of a mixture of trivalents, bivalents, and univalents, as well as multivalents greater than 3 (Fig. 1A). In other cases, nearly complete synapsis was indicated by the presence of about 10 multivalents, most appearing to be trivalents (Fig. 1B). Shortly after fertilization, the eggs resumed meiosis. Most eggs entered anaphase I around 20 min PF. Generally, replicated chromosomes (dyads) partitioned into two groups and moved toward opposite poles (Fig. 1C). We also observed that although most dyads underwent segregation at anaphase I, others remained isolated from the two segregating groups (Fig. 1D). By the time anaphase I was over, two groups of dyads had formed, each with variable numbers of chromosomes but averaging 15. The periph-

eral group became condensed during telophase I (around 30–35 min PF). Up to this stage, meiotic behavior in treated (TDCB) groups was essentially the same as in controls. Differences between the two groups appeared 35–45 min PF. In control (TD) groups, most eggs extruded the first polar body (PBI). In contrast, no polar bodies were observed in most eggs in TDCB groups. In TDCB, the peripheral group of chromosomes that was supposed to be released as PBI moved back and combined with the inner group of chromosomes, ready for meiosis II. Four typical patterns of chromosome segregation could be classified during meiosis II.

Tripolar segregation

The 30 dyads were delivered at random into three division planes in a tripolar configuration when the egg entered metaphase II (about 35–40 min PF) (Fig. 1E). During anaphase II (about 40–45 min PF), two chromatids of each chromosome detached and moved to the two adjacent poles independently (Fig. 1F). Each of the three poles received an average of 20 chromosomes from the two adjacent groups of dyads when telophase II was reached (about 45–50 min PF). However, the number of chromosomes was highly variable at each pole. After telophase II, all three groups of chromosomes became condensed. Although observations ceased before actual release of PB2, we suppose that the peripheral group of chromosomes was released as PB2 and that it is likely that PB2 sometimes contained two groups of chromosomes.

United bipolar segregation

The two groups of dyads from meiosis I united completely and aligned on a single division plane in a bipolar configuration (Fig. 1G). The sister chromatids divided and segregated to the opposite poles independently (Fig. 1H). Telophase II ended with 30 chromosomes distributed at each pole. The peripheral group became packaged and compact (Fig. 1I) and supposedly was released as PB2 after telophase II.

Incomplete united bipolar segregation

Dyads from meiosis I united completely and reorganized on a single division plane, except for a few dyads that were separated from the metaphase II division plane—"orphan chromosomes" (Fig. 1L). Subsequently, sister chromatids of dyads, which were grouped together on the division plane, separated from each other, moving to the poles independently. Concomitantly, the sister chromatids in orphan chromosomes also separated (Fig. 1M). There is no evidence that chromatids derived from orphans moved to either pole.

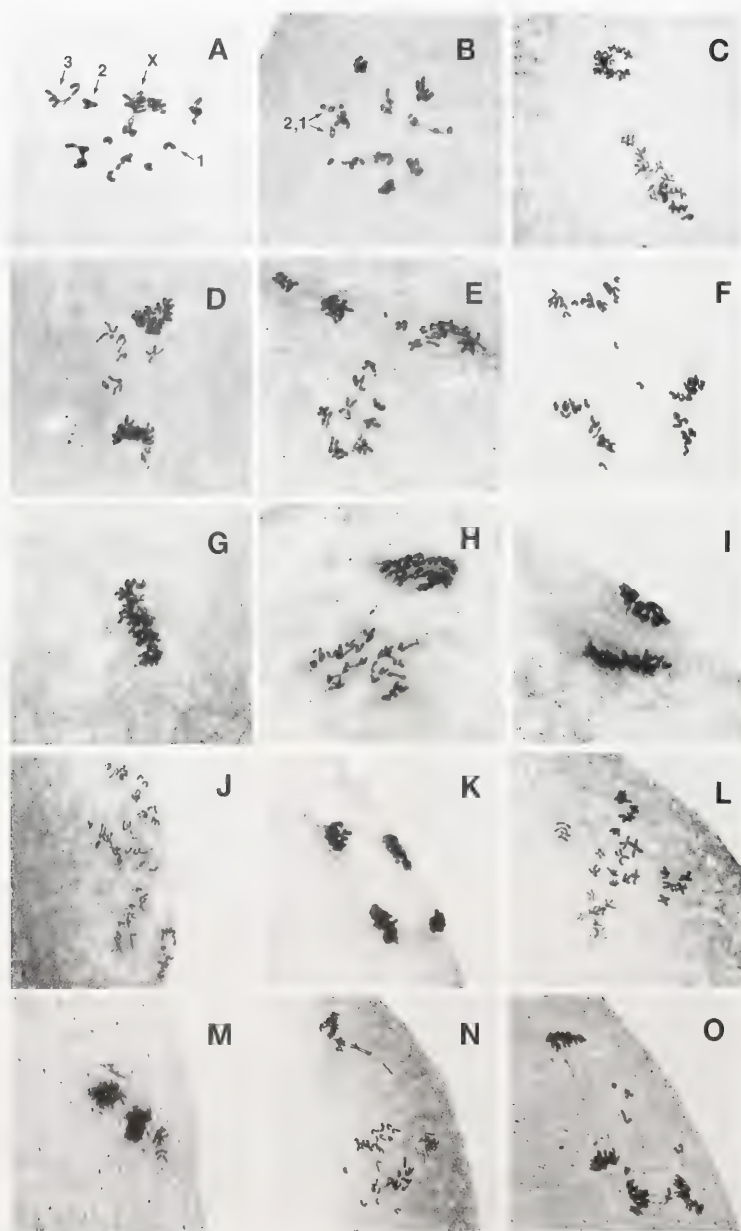


Figure 1. Chromosome segregation observed in fertilized eggs from triploid Pacific oysters, following inhibition of polar body 1. (A) mix of univalents (1), bivalents (2), trivalents (3), and multivalents ($>3 = x$) at synapsis. (B) Nearly complete synapsis, with 9 trivalents and a bi-, univalent combination (2,1). (C–D) Segregation at anaphase I. (E–F) Tripolar segregation. (G–I) United bipolar segregation. (J–K) Separated bipolar segregation. (L–M) Incomplete united bipolar segregation. (N–O) Intermediate segregation.

Rather, they remained in the area of the former metaphase II division plane.

Separated bipolar segregation

The two groups of dyads from meiosis I, rather than overlapping or uniting, entered meiosis II separately. Each group formed a bipolar configuration and then proceeded through chromosome migration (Fig. 1J). At the end of anaphase II, four groups of chromosomes were formed (Fig. 1K).

Unclassified segregation

In addition to the above four chromosome segregations, there were many intermediate segregations that could not be classified exactly. For example, we observed that one group of dyads from meiosis I went through meiosis II in a manner of bipolar segregation while the other group of dyads remained in its original state (Fig. 1N). We also found that in meiosis II, some chromosomes were left behind while others had already reached the corresponding poles and had become condensed. These aberrant and lagging chromosome movements occurred in what appeared to be either tripolar or separated bipolar configurations (Fig. 1O).

Pattern frequencies

The frequencies of the various segregation patterns were determined at telophase II by the number of maternal chromosome groups present (incomplete united bipolar segregation was identified by the presence of two groups of maternal chromosomes and some isolated chromosomes) (Table I). The majority of the treated eggs (54.5%) went through tripolar segregation.

Discussion

Completeness of synapsis in triploid Pacific oyster varied considerably, as was also found in a previous study (Guo and Allen, 1994a). Our observations demonstrated that the general configurations of synapsis that Darlington (1965) described in triploid organisms also exist in triploid Pacific oysters. According to Darlington (1965), trivalents, bivalents, and univalents can be found in the same nucleus of triploid organisms. Such modalities of chromosome pairing were also found in humans (Gosden *et al.*, 1976; Luciani *et al.*, 1978). Other studies in triploid *Allium sphaerocephalon* revealed that only two of each set of three homologous chromosomes participated in synapsis, the other remained unsynapsed (Loidl and Jones, 1986). This might be an exception to the rule in triploid organisms. Variation in the degree of synapsis in eggs from triploids might influence subsequent meiotic events following inhibition of PB1, though it was

Table I

Percentage of chromosome segregation patterns observed in fertilized eggs of triploid *Crassostrea gigas* following inhibition of PB1, in two replicates

Replicate (n)*	Chromosome segregation patterns (%)				
	Tripolar	United bipolar	Incomplete united bipolar	Separated bipolar	Unclassified patterns
1 (100)	56	13	4	2	25
2 (90)	53	11	4	3	29
mean	54.5	12.0	4.0	2.5	27.0

* Number of observations.

difficult to exactly correlate the completeness of synapsis with subsequent types of chromosome segregation. Chromosome segregation in control groups seemed to be unaffected by completeness of synapsis: most of the eggs completed two meioses in a manner similar to that seen among diploid eggs—the extra set of chromosomes segregating randomly (Longo *et al.*, 1993; Guo and Allen, 1994a). It was also found that meiotic divisions in eggs from triploid Japanese pearl oyster (*Pinctada fucata martensii*) were virtually identical to those in the diploid control (Komaru and Wada, 1994).

Our observations also suggest that the meiotic events in CB-treated triploid eggs are remarkably asynchronous. Asynchrony was observed at three levels. First, there was variation in the timing of meiosis II between the two replications, that is, between the two females. Second, among eggs from a single female, asynchrony was observed as different eggs entered meiosis I at different times. Third, asynchrony sometimes occurred among chromosomes of the same egg. For example, we observed that one group of dyads from meiosis I still remained as sister chromatids while the other group of dyads divided and moved toward opposite poles. In contrast, dyads separate synchronously during meiosis II in diploid (Guo *et al.*, 1992) and triploid (Guo and Allen, 1994b, and this study) Pacific oyster eggs.

The mechanism behind the formation of tetraploid embryos was indicated by the chromosome segregations observed in this study. United bipolar segregation was confirmed and would undoubtedly lead to the formation of tetraploids, supporting the hypothesis that tetraploids were produced in this way (Guo, 1991; Guo and Allen, 1994b). Tetraploids could also be produced by certain separated bipolar segregations. The two sets of dividing chromosomes need not have equal numbers of chromosomes so long as the anaphase/telophase II was oriented perpendicular to the egg membrane. In this case, the equational division of the dyads from both groups would yield 30 chromosomes at the periphery of the egg and 30

in the interior. Finally, the two peripheral chromosome sets would have to be released as PB2. When combined with 10 paternal chromosomes, the zygote would then contain a total of 40. Because the frequency of separated bipolar segregation was low (2.5%), it is reasonable to suppose that separated bipolar segregation accounts for few, if any, tetraploids. The other segregation patterns (tripolar segregation, incomplete united bipolar segregation, and unclassified patterns) would give rise to aneuploids with varying chromosome compositions, but rarely tetraploids. Overall, we conclude that united bipolar segregation is the major cause for the formation of tetraploids. Further support for this conclusion could be obtained by observing the correspondence between the frequency of united bipolar segregations and the percentage of tetraploidy across a number of replicates. These data are unavailable in this study.

If united bipolar segregation is the principal mechanism for tetraploid induction in Pacific oyster eggs, there is an important practical implication. Tetraploids are an important new tool in shellfish aquaculture because they can be crossed to diploids to yield populations that are 100% triploid (Guo *et al.*, 1996). In turn, triploid progeny are a valuable product for growers because of the value-added benefit of their reproductive sterility (Allen, 1988). Therefore, the production of tetraploid broodstock for use in hatcheries will be increasingly important. This study indicates that the high mortality among larvae from spawns to make tetraploids (*i.e.*, $3n \times 2n$ crosses) (Guo and Allen, 1994b, and unpubl. data) is a normal and inescapable consequence of this technique for making tetraploids—the cost of doing business. Only about 12% of all segregations in PB1-inhibited triploid eggs can be expected to yield tetraploid embryos and larvae. The rest would consist of a few euploids and various aneuploids, some of which will be viable (Guo and Allen, 1994a, b). Of course, only euploids and less severe aneuploids will survive through metamorphosis. Overall, the percentage of embryos that receive viable chromosome complements following inhibition of PB1 in triploid eggs is small. Of particular interest is that the frequency of united bipolar segregations observed here (mean: 12%) is almost double the frequency observed in PB1-inhibited diploid eggs (Guo *et al.*, 1992).

The patterns of chromosome segregation in eggs from triploids, following inhibition of PB1, were similar to those in diploid eggs following the same treatment, which implies that similar mechanisms underlie the segregation of chromosomes during meiosis II. We speculate that the behavior of centrioles is ultimately responsible for these patterns of chromosome segregation. Centrioles are intimately involved in the formation of meiotic spindles in *Mytilus edulis* (Longo and Anderson, 1969) and *Spisula solidissima* (Longo and Anderson,

1970). The role of centrioles in establishing multipolar meiotic apparatuses was also suggested in a recent investigation concerning the effect of cytochalasin B during meiotic maturation of diploid eggs from *Crassostrea gigas* (Longo *et al.*, 1993). In that study, inhibition of PB1 caused the formation of a tripolar spindle. The authors suggested that the centrioles normally extruded with the first polar body would participate in the organization of one pole of the tripolar spindle. The other centriole, normally remaining in the egg, divided and was responsible for the organization of two poles of the tripolar spindle. It is likely that the same mechanism underlies the formation of tripolar configurations observed in this study. Furthermore, the united bipolar segregation could be the consequence of either the dysfunction of the peripheral centrioles or the nondivision of inner centrioles; separated bipolar segregation could arise from the division of both the resident and peripheral centrioles. However, these hypothetical mechanisms by which chromosomes segregate during meiosis II following inhibition of PB1 remain to be proved in future studies.

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Effects of Spatial Distribution and Reproductive Biology on *in situ* Fertilization Rates of a Broadcast-Spawning Invertebrate

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Abstract. *In situ* fertilization was examined in the gorgonian *Pseudoplexaura porosa* during 1994 and 1995 spawning events in the San Blas Islands, Panama, to assess spatial and temporal variation in fertilization success and to determine whether *in situ* fertilization was sperm limited. Fertilization rates did not differ significantly between years (60% vs. 55%), but monthly means were significantly different, ranging from 22% to 66%. Fertilization rate varied among days, ranging from 0 to 85%; 80% of this variability was explained by daily variation in the number of colonies that spawned. A weighted average of *in situ* fertilization rates suggests that 67% or more of spawned eggs are fertilized in nature. Sperm limitation did not occur on the nights when most of the colonies synchronously spawned and when most of the eggs were released. Eggs collected downstream of the population often had higher fertilization rates than eggs collected either adjacent to their source colony or eggs collected in the middle of the population, which indicates that in dense populations, eggs may have multiple opportunities to be fertilized. Traits such as highly synchronous spawning, high fecundity, large egg size, large polyps, and large colonies directly and indirectly enhance *P. porosa* gamete production and fertilization. These life-history traits reduce the effects of gamete dilution during spawning events and thus decrease the importance of sperm limitation in the population dynamics of *P. porosa*.

Introduction

Whether or not fertilization rates constrain the reproductive success of free-spawning organisms has become a subject of increasing interest in the last 10 years (see Levitan, 1995, for review). Despite a growing number of studies on fertilization rates, the questions still remain whether low fertilization rates are common, whether low fertilization rates are caused by sperm limitation, whether fertilization rates limit reproductive success, and whether morphologies and behaviors that enhance fertilization act as constraints on the life-history evolution of broadcast-spawning taxa. We examined the fertilization rates of the Caribbean gorgonian *Pseudoplexaura porosa* in an effort to address these questions.

The conclusion that fertilization success is an important factor controlling the overall reproductive success of broadcast-spawning species comes from three types of data: observations of low fertilization rates during natural spawning events; experimental manipulations indicating that sperm density can limit fertilization success; and hydrodynamic models of gamete dilution. Low fertilization rates have been reported in taxa ranging from coelenterates to fishes (Petersen, 1991; Oliver and Babcock, 1992; Babcock and Mundy, 1992; Babcock *et al.*, 1992; Petersen *et al.*, 1992; Brazeau and Lasker, 1992; and Levitan, 1995). Moreover, fertilization rates are highly variable: even species with high average fertilization rates may exhibit cases of very low fertilization (*e.g.*, Sewell and Levitan, 1992), and conversely, species with low fertilization rates sometimes demonstrate high rates when observed over many days (*e.g.*, *P. kuna*; Lasker *et al.*, 1996).

When they occur, low fertilization rates have generally

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been attributed to sperm limitation. The only experimental demonstration of sperm limitation during natural spawning events is that of Lasker *et al.* (1996), who found that incubating naturally spawned eggs with supplemental sperm enhanced the fertilization rates of *Plexaura kuna*. Field experiments that report decreasing fertilization rates with distance downstream from spawning males again suggest that sperm density limits fertilization success (Pennington, 1985; Yund, 1990; Levitan, 1991; Levitan *et al.*, 1991, 1992; Babcock *et al.*, 1994; Brazeau and Lasker, 1992; Oliver and Babcock, 1992; Benzie *et al.*, 1994; Benzie and Dixon, 1994; Yund and McCartney, 1994; Levitan, 1995; Levitan and Young, 1995; Lasker *et al.*, 1996; Coma and Lasker, 1997). Although the effects of the downstream dilution of sperm can be lessened by synchronous spawning, among many species only a small proportion of the population participates in natural spawning events (Randall *et al.*, 1964; Mosher, 1982; Pennington, 1985; Michin, 1987; Levitan, 1988; Pearse *et al.*, 1988; McEuen, 1988; Babcock and Mundy, 1992; Babcock *et al.*, 1992; Gladstone, 1992; Beiring and Lasker, unpubl. data).

Hydrodynamic models and measurements of turbulent diffusion and the dilution of sperm also predict that fertilization rates should be low under many circumstances (Denny, 1988; Denny and Shibata, 1989; Lasker and Stewart, 1993; Levitan and Young, 1995; Lasker and Kapela, 1997). However, the predictions of those models are dependent on variables such as the rate of gamete release and net flow, and several authors have reported cases in which high fertilization rates are predicted (Denny *et al.*, 1992; Benzie *et al.*, 1994; and Levitan and Young, 1995).

Sperm limitation is not the only cause of low fertilization rates. For example, Levitan (1996a) reports differences in quality among sea urchin eggs, and similar effects have been observed among gorgonians (Lasker, unpubl. data). In addition, Mead and Denny (1995) have identified hydrodynamic conditions that interfere with sperm-egg interactions regardless of the concentration of sperm. Thus the commonness and causes of fertilization limitation are unclear and may be species specific.

Caribbean gorgonians are among the few taxa in which fertilization rates can be predictably and directly measured during natural spawning events. Many gorgonians exhibit synchronous spawning (Kinzie, 1970; Brazeau and Lasker, 1989, 1990), and the large eggs released by octocorals can be collected from the water column (Lasker *et al.*, 1996) or from the surface of the colony (Brazeau and Lasker, 1992). Although there are some overlaps between species in the timing of spawning, many taxa spawn on different days or at different times of day, allowing the collection of monospecific groups of gametes. The gorgonians studied to date have the lowest

fertilization rates that have been measured *in situ* (*Briaricum asbestinum* <0.01%–6%, Brazeau and Lasker, 1992; *Plexaura kuna* on average 26.4%, data from Lasker *et al.*, 1996). Lasker *et al.* (1996) also provided data on fertilization success in *Pseudoplexaura porosa*, noting that high fertilization rates (including some >80%) were more common in *P. porosa* than in *P. kuna*. These species appeared to differ in their fertilization rates and in the importance of sperm limitation. In this paper we expand our observations of *P. porosa* and show how reproductive strategy affects fertilization success in this species.

Material and Methods

Fieldwork was conducted at Korbiski reef, a patch reef located near the Smithsonian Tropical Research Institute (STRI) field station in the San Blas Islands, Panama. *Pseudoplexaura porosa* is a gonochoric broadcast spawner with a 1:1 sex ratio; it spawns in highly predictable events that occur shortly after sunset after the summer full moons (Lasker *et al.*, 1996; Ross and Lasker, unpubl. data). *P. porosa* is the second most abundant gorgonian on Korbiski reef (Lasker *et al.*, 1988) and, because of the size of its colonies (up to 250 cm in height), one of the dominant members of the benthos. The *P. porosa* population at Korbiski is most dense in a 150-m² area on the eastern side of the reef along a channel that separates Korbiski from an adjacent reef. The colonies at Korbiski were labeled and mapped. All colonies taller than 50 cm were measured, and a fragment was collected for sex identification.

Field collections were made during all spawning events in 1994 (June to September) and during June, July, and August in 1995. Eggs were collected from the water column shortly after they were released from colonies. Scuba divers positioned downstream of either specific colonies or the whole population collected the eggs (700 μ m in diameter) in 60-ml plastic syringes. The syringes containing the collected eggs were brought to the STRI field station and after 0.5–1.5 hours all eggs were counted, placed in 120-ml polypropylene specimen cups, and incubated with seawater that had been collected prior to the start of spawning (sperm-free seawater). Containers were suspended from the field station dock to maintain temperature and provide some stirring. After 12 h the developing embryos were counted with the aid of a stereomicroscope. That value was used as the estimator of the number of eggs that had been fertilized. Results are expressed as percent fertilization and were arctan transformed prior to statistical analysis. Lasker *et al.* (1996) discussed the biases inherent to these procedures and concluded that the procedures did not introduce a large systematic effect, but they did note that ob-

served fertilization rates may slightly underestimate the true fertilization rate.

In 1994 we assessed overall fertilization rates as well as spatial variation in fertilization. Overall fertilization rates were determined from eggs collected 5 m downstream of all of the *P. porosa* colonies on each night of spawning. The exact position of the diver collecting the eggs was based on the prevailing current. Lasker *et al.* (1996) have previously shown that in the absence of downstream male colonies, fertilization rates do not differ over the range of 1 to 5 m from a spawning female. Farther downstream eggs became too dilute to be collected in a significant number. To determine where eggs are fertilized, eggs were also collected either at a position 0.5–1 m downstream from a female colony (June 1994) or from the center (labeled C in Fig. 1) of the population (July 1994). Positional effects were also studied in September 1994, when eggs were collected both upstream and downstream of a male colony.

In 1995 the sampling program was designed to measure overall fertilization rates and test for the presence of sperm limitation in the field. All measures of *in situ* fertilization were carried out downstream of the whole population. We also monitored the number of female colonies spawning by examining each female colony on the reef every 15 min and recording the start and end of spawning for each colony. Male spawning was not readily observed; to verify that it had occurred, water samples were collected adjacent to arbitrarily selected male colonies and sperm densities were determined by means of a modification (Swain *et al.*, 1997) of the acridine orange direct count technique (Hobbie *et al.*, 1977).

An InterOcean S4 current meter was used to estimate the speed and direction of water flow. The current meter was placed within the population, 1.5 m above the bottom, at the height of most colonies. The S4 meter sampled continuously at 0.5-s intervals starting 15 min before spawning and continuing until egg release had ended.

Sperm limitation was tested in enrichment experiments similar to those described by Lasker *et al.* (1996) for *Plexaura kuna*. One experiment was conducted during each month of spawning in 1995 (3 nights). Eggs were collected on the reef in syringes as described above. Two divers collected side by side, downstream of the whole population. During all three experiments the current ran to the south, and the divers were located at position S (Fig. 1). The eggs, which float, were captured about 3 m above the substratum in the 6-m water column. Collection began at the start of the spawning event and continued for about 60 min, yielding 13 to 15 pairs of samples (55–60 eggs per sample). The samples were then transported to the STRI field station where the paired samples were pooled. From each group of eggs, 50 were

incubated with sperm-free water and another 50 with water from an aquarium containing a male colony. As in the field samples, eggs were assayed the following morning to determine the proportion fertilized. As a positive control, 50 virgin eggs were incubated in water from the male aquarium colony. Three replicate controls were set up at the beginning and again at the end of the enrichment process (about 25 min later). The colony explants used to obtain virgin eggs and sperm were collected from Korbiski reef 2–3 days before spawning. Explants were maintained in 18-liter aquaria, and the water was vigorously exchanged every 2 h between 0600 and 1800 h and then once in the evening (2200–2400 h). Water samples from the male tank were collected at the time of the experiment, and sperm density was estimated following the protocol of Swain *et al.* (1997).

Results

Spawning

Spawning in *P. porosa* was highly synchronized in a restricted 2–4 day period that started 5–6 days after the full moon each month from June to September. Scattered observations from the summer months of 1987 through 1995 (36 observation nights) showed that all spawning events occurred 5–10 days after the full moon. Monthly spawning events were characterized by 1–2 nights of intense spawning and 1–2 nights of weak spawning. Usually, the first and the last nights had weak spawning. Most colonies started spawning between 1815 and 1915 h and ended between 1915 and 1945 h. Differences in the timing of egg release between specific colonies were consistent between months and years. Female colonies began spawning by releasing eggs at a low rate for about 10 min. Egg release steadily increased over 15–30 min and then rapidly decreased at the end of the spawning. Sometimes female colonies had two peaks of intense spawning during a single night.

Spatial distribution of colonies

In the study area the density of colonies taller than 50 cm was 0.1 colonies per square meter. The population of colonies with identifiable gonad consisted of six male colonies (height = 125–230 cm, mean = 194 cm), and six female colonies (height = 101–201 cm, mean = 142 cm). Samples collected at either of the two downstream collecting positions (N and S; Fig. 1) had similar mean distances to the six male colonies (N—distance = 4–19.7 m, mean = 13 m; S—6–23 m, mean = 14.8 m). Collections at the central position (C; Fig. 1) always excluded half of the male population as a result of the unidirectional flow regime. The center collection site had a similar mean distance to male colonies (center position

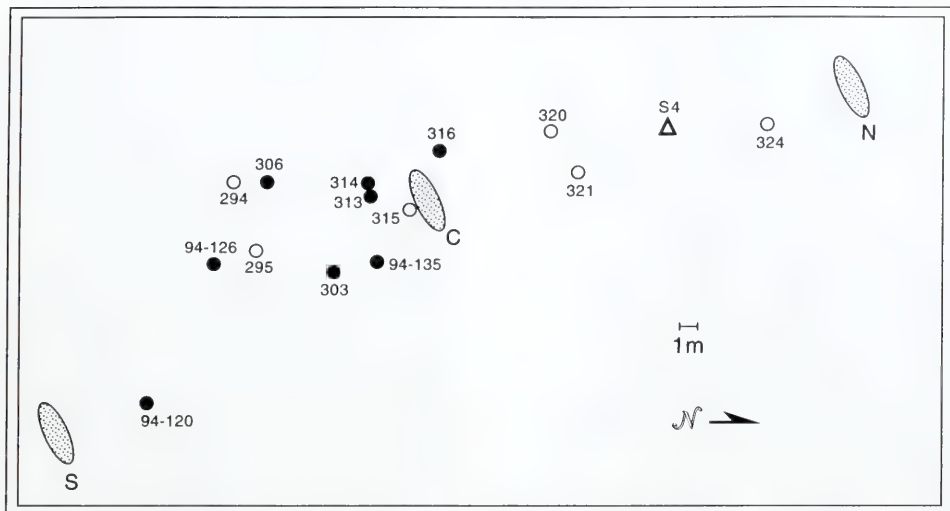


Figure 1. Map of *Pseudoplexaura porosa* colonies (taller than 50 cm) at Korbiski reef, San Blas Is., Panama. Female colonies (filled circles), male colonies (empty circles), and S4 current meter (triangle). Collections were made at positions downstream of the entire population (N or S) and one position in the center of the population (C).

with current from the north—3 colonies at a distance of 3.4–10 m, mean = 5.6 m; center position with current from the south—3 colonies at a distance of 1.8–7 m, mean = 5.1 m).

Variability in fertilization

In situ fertilization ranged between 0% and 98%. The majority (66%) of the downstream samples had levels of

fertilization above 50%. The modal fertilization rate was 80%–90% in both years (Fig. 2). Fertilization rates did not differ significantly between years, but did vary significantly between months and days (Tables I and II). The lowest monthly mean was observed in September. Fertilization was high on at least one night during each month (Table II). Annual fertilization success during 1995 was estimated by weighting the fertilization rate of each night by the number of female colonies observed spawning (Table II; Fig. 3), yielding an estimated fertilization rate of 67%. Sperm release was not visible in the field, and measures of sperm density were highly vari-

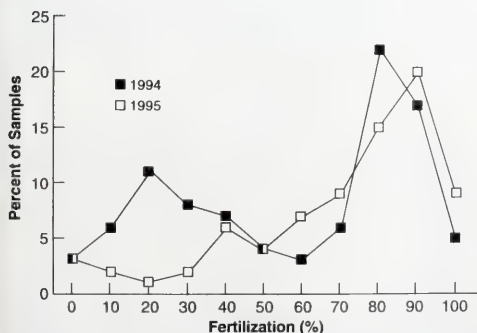


Figure 2. Frequency distribution of fertilization rates among all samples of *Pseudoplexaura porosa* eggs from all nights.

Table I

Nested analysis of variance of fertilization rates of *Pseudoplexaura porosa* between years, among months, and among days (months nested in years, and days nested in months)

Source	df	SS	MS	F	P
Year	1	0.004	0.004	0.32	0.5717
Month	6	19.87	3.31	281.27	<0.0001
Day	8	14.65	1.83	155.61	<0.0001
Error	152	1.79	0.12		

df, degrees of freedom; SS, sums of squares; MS, mean square; F, F ratio; P, probability.

Table II

In situ fertilization rate of *Pseudoplexaura porosa* at Korbyski

Date	No. samples	No. eggs collected	Fertilization rate*			No. ♀ colonies spawning	Sperm density†	Flow	
			Day	Month	Year			cm/s	dir (°N)
1994									
29.VI	16	772	71			—	—	—	
30.VI	19	1023	46			—	—	—	
1.VII	12	367	9	47		—	—	—	
28.VII	16	724	31			—	—	—	
29.VII	21	507	73			—	—	—	
30.VII	31	1757	83	66		—	—	—	
28.VIII	17	615	75			—	—	—	
29.VIII	1	46	57	66	60	—	—	—	
25.IX	9	473	27			—	—	7.0	344
26.IX	12	602	16	22		—	—	13.3	352
1995									
18.VI	11	530	48			3	—		
19.VI	26	1300	82			5	—	—	
20.VI	8	481	30	53		1	190	11.2	335
17.VII	3	132	0			1	—		
18.VII	12	709	69			6	1780		
19.VII	26	1300	83	51		6	5200	13.3	148
16.VIII	8	448	40			4	60		
17.VIII	30	1500	85	63	55	5	2370	3.1	51

* Daily, monthly, and annual mean percentages.

† Cumulative sperm/ml.

able, ranging from 0 to 2700 sperm/ml for samples collected from the same male. There was no correlation between fertilization and cumulative sperm release for those nights. Because we could not see sperm coming from the colony, it is unclear whether the variation in sperm density reflects sperm release or the positioning of the sample bottle relative to the "plume" of sperm being

released. Assuming that gamete release must be somewhat synchronous between sexes, we also used the number of spawning females as an indicator of the number of spawning male colonies. The number of spawning female colonies explained 80% of the variance in fertilization success observed between nights (Fig. 3).

Spatial variability was examined in experiments using three different designs. Fertilization rates among eggs collected close to the colonies versus those collected downstream of the whole population changed significantly, but the effect varied between nights (Table III). There also was a significant day $\times$ position interaction.

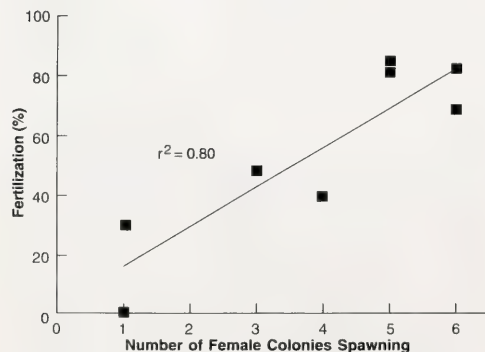


Figure 3. Relationship between number of female colonies spawning and mean daily fertilization rates for *Pseudoplexaura porosa* eggs.

Table III

Analysis of variance testing for fertilization rates of *Pseudoplexaura porosa* collections carried out close to a spawning female colony and downstream of the population (Position), and among days (Day)

Source	df	SS	MS	F	P
Day	2	1.11	0.56	26.86	<0.0001
Position	1	0.10	0.10	4.81	0.0348
Day $\times$ Position	2	0.31	0.16	7.51	0.0019
Error	36	0.75	0.02		

df, degrees of freedom; SS, sums of squares; MS, mean square; F, F ratio; P, probability.

On two nights, the fertilization rate 1 m from a female colony was lower than the downstream fertilization rate (29 June 1994: 26% at 1 m vs. 71% downstream; 30 June 1994: 3% at 1 m vs. 9% downstream), but on a third night, fertilization close to the female colony was higher than fertilization downstream (63% at 1 m vs. 46% downstream). On 30 June 1994, a third diver also collected samples immediately downstream of the spawning female. The difference between the two collections made at the female colony, 38% and 3%, is indicative of the spatial variability in fertilization rates. Given the variation between divers at the colony, it is not surprising that there were no significant differences between the samples collected at the colony and those collected downstream of the population (ANOVA, $F_{2,9} = 3.02$, $P = 0.099$). However, it is also important to note that relatively few samples were collected that night, which was the last night of spawning.

On 29 and 30 July 1994, a position effect was observed between collections from the center of the population and those downstream. Fertilization was higher at the downstream position than at the position in the center of the population (two-way ANOVA: $F_{1,35} = 33.10$, $P < 0.0001$; Fig. 4). Another experiment (26 Sept 1994) compared the fertilization rates between eggs collected immediately upstream of a male colony and those collected downstream of the male. There was total failure upstream and 16% fertilization downstream (one-way ANOVA, $F_{1,11} = 117.22$, $P < 0.0001$). The low fertilization rate probably occurred because this was the last night of spawning in September and few colonies released gametes. Examination of polyps of colonies sampled 3 days before the September spawning showed that the colony used in the experiment was the only male colony with mature spermaries.

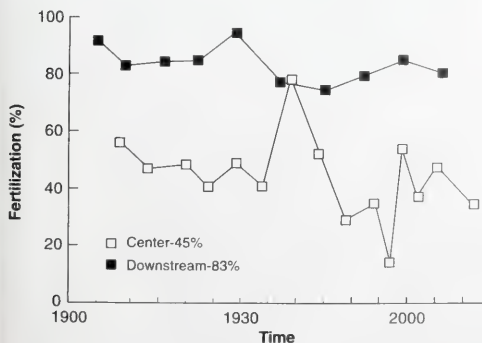


Figure 4. Proportion of *Pseudoplexaura porosa* eggs fertilized in individual samples collected on 29 July 1995 at the center of the population (center) and downstream of the entire population (downstream). Mean fertilization rate at each collection site is also shown.

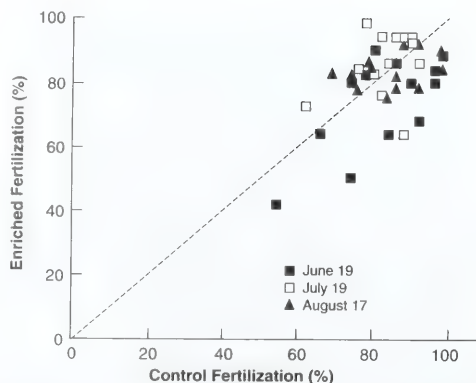


Figure 5. Fertilization rates among in situ samples of *Pseudoplexaura porosa* eggs incubated with (enriched) and without (control) additional sperm.

Sperm-limitation experiments

Enrichment experiments were conducted on a single evening each month during the 1995 spawning events. To ensure an adequate supply of eggs, the enrichment experiments were conducted on the nights with the greatest density of eggs in the water column (19 June, 19 July, and 17 August). Those were the days on which the greatest number of spawning females were observed and *in situ* fertilization rates were over 80% (Table II). There were no significant differences between field fertilization and sperm-enriched samples (paired *t* test, $t = 1.39$, $df = 39$, $P = 0.174$; Fig. 5), indicating that sperm limitation did not occur on these three nights. Control incubations carried out in the laboratory at the beginning of the experiment always had fertilization rates higher than 80% (81%–82%) and did not differ from controls initiated at the end of the experiment (two-way ANOVA, $F_{1,8} = 1.25$, $P = 0.296$).

The limited data on flow speed suggests that flow over the range of observed speeds had little impact on fertilization. For instance, fertilization was greater than 80% on both 19 July and 17 August 1995 despite the fourfold difference in current speed on the two nights (Table II).

Discussion

Fertilization success in *P. porosa* was often substantially less than 100%, and *in situ* rates were often lower than those reported for other broadcast-spawning taxa (Petersen, 1991; Oliver and Babcock, 1992; Babcock and Mundy, 1992; Babcock *et al.*, 1992; Sewell and Levitan, 1992; Petersen *et al.*, 1992). As we previously noted (Lasker *et al.*, 1996), this suggests that *P. porosa* fertiliza-

tion rates are sperm limited at times. There also was a general correspondence between sperm release in 1995 and fertilization rates (Table II), but our data also suggest that most eggs are released under conditions in which sperm are not limiting.

First, we determined that 67% of eggs released over the entire spawning event are fertilized. To calculate average fertilization rates, we weighted the fertilization rate from each night by the number of colonies releasing eggs. The number of eggs being released per colony also varies among nights, and it probably peaks on the same nights that the greatest number of colonies spawn. Therefore, our weighted averages probably underestimate fertilization.

Second, we did not detect any evidence of sperm limitation in sperm-enrichment experiments, which were conducted on the nights on which most spawning occurs. That 100% fertilization was never observed in those experiments may be a function of biases introduced by the sampling and incubation techniques (Levitan, 1995; Lasker *et al.*, 1996), but those biases would have affected both the controls and treatments. The failure to see enrichment in samples that yielded only 50% fertilization rates (Fig. 5) suggests that eggs in those particular incubations were less capable of developing, due either to natural variation in gamete quality, or to the experimental technique. In either case the conclusion that sperm were not limiting is not affected.

Spatial variability in fertilization

Lasker *et al.* (1996) showed that eggs collected within 1 m of a colony of *Pseudoplexaura* sp. had similar fertilization rates to eggs collected downstream. Presumably sperm are continuously diluted as they are transported downstream, so eggs released into the water column interact with ever decreasing concentrations of sperm. Therefore, eggs that are not rapidly fertilized will have ever decreasing probabilities of being fertilized. Of course, downstream additions of sperm can affect fertilization. Fertilization rates that we measured did not consistently vary between eggs collected within a meter of the colony and those collected downstream of the entire population, but collections made in the center of the population regularly underestimated the fertilization success of the population. Whether fertilization increased downstream of individual female colonies was probably a function of the behavior of the nearby male colonies, and that effect appears to have varied among nights and colonies. Distance had a positive effect on fertilization when downstream transport enabled eggs to travel past additional male colonies.

Temporal variability in fertilization

Fertilization was very similar in both years. The significant difference between months was mainly due to the low fertilization rate in September. Monthly variation can probably be attributed to gamete release (Ross and Lasker, unpubl. data) or to monthly changes in fertilization kinetics similar to that observed in *Plexaura kuna* (Lasker and Stewart, 1993 and unpubl. data) and in *Acanthaster planci* (Benzie and Dixon, 1994).

Individual size and local population density have significant effects on fertilization rates among echinoderms (Levitan, 1991; Levitan and Young, 1995), presumably through their effects on sperm density in the water column. Sessile organisms cannot aggregate, but synchronization of spawning increases the likelihood of fertilization. The importance of spawning synchronization is evident in *P. porosa* because most of the variance in fertilization among nights was explained by the number of spawning colonies. Although our measure of sperm density did not correlate with fertilization rates, the correlation between fertilization and the number of spawning colonies is likely a reflection of the density of gametes in the water column. This is well illustrated in the 26 September 1994 data which show that when few colonies spawned, most eggs were not fertilized. Conversely, on nights when most of the spawning occurred, fertilization rates were highest and the enrichment experiments revealed no evidence of sperm limitation.

Reproductive strategy and fertilization success

Fertilization rates were low on some nights and sperm limitation probably did occur on those nights; nevertheless, the overall effect of sperm limitation on fertilization success in *Pseudoplexaura porosa* may be low. This result is in marked contrast to the low *in situ* fertilization rates reported for *Briareum asbestinum* (Brazeau and Lasker, 1992) and *Plexaura kuna* (Lasker *et al.*, 1996). Furthermore, the sperm-enrichment experiments failed to detect sperm limitation with *P. porosa*, whereas the results of identical experiments at the same study site strongly suggested sperm limitation with *P. kuna* (Lasker *et al.*, 1996).

Although few octocorals have been examined, we can compare gorgonians for which there are data to assess the effects of life-history traits on fertilization success. At Korbiski and at many sites at which it is common, *P. porosa* dwells at relatively low density in habitats with fast (>10 cm/s) unidirectional currents. Models of fertilization processes predict low fertilization rates under these conditions (Levitan and Young, 1995). However, fertilization rates are higher in *P. porosa* than in other gorgonians, a feature that is probably explained by traits

such as gamete production, fertilization kinetics, spawning pattern, egg size, polyp size, and colony growth rate.

Gamete density—and in particular sperm density—is one of the principal factors determining fertilization success (egg density has only a small effect on fertilization: Lillie, 1915; Vogel *et al.*, 1982; Levitan *et al.*, 1991). *P. porosa*, which is not sperm limited, has spermaries that are an order of magnitude greater in volume than those of *Plexaura kuna*, which is sperm limited (Lasker *et al.*, 1996); and *P. porosa* has the largest spermaries ($0.8 \text{ mm}^3/\text{polyp}$; Ross and Lasker, unpubl. data) found among the studied gorgonian species (*Briareum asbestinum*, $0.05\text{--}0.15 \text{ mm}^3/\text{polyp}$; Brazeau and Lasker, 1990; *Paramuricea clavata*, $0.4 \text{ mm}^3/\text{polyp}$; Coma *et al.*, 1995b; *Plexaura kuna*, $0.07 \text{ mm}^3/\text{polyp}$; Lasker and Stewart, 1993; *Plexaura flexuosa*, $0.05 \text{ mm}^3/\text{polyp}$; Beiring and Lasker, unpubl. data).

Spawning synchrony also plays an important role in determining gamete density and fertilization success (Thorson, 1946; Harrison *et al.*, 1984; Sewell and Levitan, 1992). *P. porosa* spawning was limited to about 1 h on each of 4 nights during each of 4 months. Furthermore, most spawning occurred on only 2 of the 4 nights each month. A short spawning period should generate higher gamete concentration than a long spawning period. Spawning over several months allows greater total gamete production, whereas spawning over several days within each month increases the chances of spawning on nights with favorable conditions for fertilization.

Preliminary experiments (unpubl. data) have shown that, in addition to producing many sperm, at least some gorgonians produce gametes that are able to fertilize a large percentage of eggs ($>70\%$) at low sperm concentration (10^2 sperm/ml). This suggests that the fertilization kinetics of *P. porosa* could yield higher levels of fertilization at lower sperm concentration than can be achieved by other broadcast-spawning taxa (sea urchins—Vogel *et al.*, 1982; Levitan *et al.*, 1991; corals—Oliver and Babcock, 1992).

Levitan (1993, 1996a,b) has argued that large eggs present a larger target for sperm and thus increase the probability of fertilization. *P. porosa* eggs are among the largest ($700\text{--}750 \mu\text{m}$, Ross and Lasker, unpubl. data) observed in gorgonians (*Corallium rubrum*: $300\text{--}330 \mu\text{m}$, Vigni, 1970; *Muricea californica*: $700 \mu\text{m}$, *M. fruticosa*: $600 \mu\text{m}$, Grigg, 1977; *Plexaura homomalla*: $315\text{--}640 \mu\text{m}$, Martin, 1982; *Plexaura kuna*: $500\text{--}600 \mu\text{m}$, Brazeau and Lasker, 1989; *Briareum asbestinum*: $600\text{--}900 \mu\text{m}$, Brazeau and Lasker, 1990; *Paramuricea clavata*: $400\text{--}500 \mu\text{m}$, Coma *et al.*, 1995a). *P. porosa* polyps are the largest among gorgonian species in which reproduction has been studied. This trait is important because a large proportion of the fully mature polyp

is occupied by gonad, so polyp size may limit gamete production.

Large colony size also contributes to total gamete release, and *P. porosa* colonies are large relative to other reef gorgonians. *P. porosa* also has the fastest growth rate observed among studied gorgonian species (up to $20 \text{ cm}/\text{year}$, unpubl. data). Energetic constraints (Harrison and Wallace, 1990) or polyp internal space may limit colony reproductive output. Regardless of the proximal factors controlling growth and reproduction, *P. porosa* colonies inhabit environments that enable them to produce large colonies through fast growth; this, in turn, promotes the production of a large number of gametes at a single point.

P. porosa is common in areas of high flow. Flow affects processes such as feeding, metabolic rate, fertilization, fragmentation, and survival. Although flow may enhance traits such as feeding, and thus the energy balance of a colony, high flow probably reduces the rate of fertilization. The adverse effects of flow rate on fertilization have been observed in many species (Pennington, 1985; Levitan *et al.*, 1992; Petersen *et al.*, 1992; Levitan and Young, 1995; Levitan, 1996a; Coma and Lasker, 1997). However, on the nights we monitored fertilization, the number of spawning colonies appeared to have a greater effect than flow. For instance, on the nights of 19 July and 17 August 1995, a large percentage of the population released eggs and fertilization was over 80% , yet flow on the two nights varied fourfold (Table II). *P. porosa* life-history traits probably reduce the effects of flow on fertilization and thus reduce the importance of sperm limitation.

The Korbiski population produced an estimated 2.1×10^6 eggs ($839 \text{ branches} \times 50 \text{ cm/branch}$ [minimum estimation of reproductive tissue per branch] $\times 70$ polyps/cm [$\text{SD} = 14$, $n = 10$] $\times 4.3 \text{ eggs/polyp}$ [Ross and Lasker, unpubl. data]). The six mature colonies of the studied population were distributed over an area of 150 m^2 ; if 67% of all eggs were fertilized, then $56,000$ embryos/ m^2 were produced annually. This estimate of larval production is similar to that for *Eunicella singularis* ($60,000 \text{ larvae}/\text{m}^2$; Theodor, 1967). The production of eggs in *P. porosa* ($84,000 \text{ eggs}/\text{m}^2$) at Korbiski is an order of magnitude smaller than that documented for *Paramuricea clavata* ($730,000 \text{ eggs}/\text{m}^2$; Coma *et al.*, 1995b). *P. clavata* has a reproductive biology (Coma *et al.*, 1995a) similar to that of *B. asbestinum*, and *B. asbestinum* has fertilization rates that are an order of magnitude lower than those of *P. porosa* (Brazeau and Lasker, 1992).

The life history of *P. porosa* is affected by a wide variety of factors. For instance, polyp size affects feeding as well as gonad volume; egg size may affect larval longevity; growth rates affect maturation and relative probability

ities of survival. It is simplistic to argue that the life history of *P. porosa* is built around fertilization success alone, because selection to increase fertilization rate will be balanced by selection on other life-history traits and limited by morphological constraints (Levitán, 1995). Nonetheless, it is clear that many *P. porosa* life-history traits are successful at enhancing fertilization compared with that of species without such traits. Although all eggs were not fertilized and fertilization rates were variable, sperm limitation was not regularly observed. Sperm limitation probably plays a lesser role in the population ecology of *P. porosa* than of other gorgonians.

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Larval Shell Muscles in the Abalone *Haliotis kamtschatkana*

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Abstract. I used light and electron microscopy to investigate shell-attached muscles in larvae of *Haliotis kamtschatkana* Jonas, 1845, because an early description of these muscles in *H. tuberculata* by Crofts (1937, 1955) has featured prominently in theories about gastropod evolution. Larval shell muscles in *H. kamtschatkana* can be grouped into two categories. The first category consists of the larval retractor muscle (LRM) and the accessory larval retractor muscle (ACC); these are striated muscles in which myofilaments begin differentiating before the head and foot rotate relative to the protoconch (this rotation is known as *ontogenetic torsion*). Collectively, these muscles ultimately insert on tissues within the larval head and mantle, but the ACC and mantle fibers of the LRM degenerate as metamorphic competence is achieved. The second category consists of two nonstriated pedal muscles that differentiate after cephalopodial rotation. The left pedal muscle is anchored on the back of the protoconch, to the left of the shell-attachment plaque for the LRM. It projects into the foot primarily, but also gives rise to muscle slips extending into the mantle fold. The right pedal muscle is anchored on a calcareous septum secreted along the visceral rim of the protoconch. The new data force a reconsideration of the ancestral homologues of larval shell muscles in abalone, because Crofts may have misidentified the accessory larval retractor muscle as a precursor of one of the later pedal muscles.

Introduction

The form and development of shell-attached muscles in vetigastropods and Patellogastropods, which I will call

'archaeogastropods' (see Hickman, 1988; Haszprunar, 1993), have had considerable impact on theories about early gastropod evolution. Studies by Crofts (1937, 1955) on muscle morphogenesis in *Haliotis tuberculata* and several other archaeogastropods have been particularly influential. Crofts identified two shell muscles in larvae of *H. tuberculata*: the larval (or velar) retractor muscle and the future columellar muscle. She suggested that the precocious differentiation of the larval retractor muscle relative to the columellar muscle initiates the mechanical twisting of the developing larval body known as ontogenetic torsion. Crofts (1937, 1955) also indicated that the pair of larval muscles become the two adult shell muscles in *H. tuberculata*, a condition viewed as primitive within a molluscan class in which most members have only one adult shell muscle. The diagrams in Figure 1A, B depict Crofts' (1937, 1955) interpretation of the larval shell muscles in *H. tuberculata* and several other archaeogastropods during an early and late stage of premetamorphic development.

Crofts' (1937, 1955) descriptions endorsed and extended the hypothesis that the two shell muscles in archaeogastropod larvae, and their postmetamorphic derivatives, are bilateral homologues that have lost their ancestral symmetry (Garstang, 1929). The two are presumed to be descendant remnants of serially duplicated, symmetrical pairs of dorso-ventral shell muscles as retained by extant monoplacophorans and chitons (Knight, 1952; Stasek, 1972; Wingstrand, 1985). This hypothesis has influenced interpretations of paleontological data (see Yochelson, 1967; Harper and Rollins, 1982; Runnegar and Pojeta, 1985) and speculations about evolutionary diversification of shell muscles among extant larval and adult gastropods (Fretter and Graham, 1962; Fretter, 1969; Haszprunar, 1985).

Despite the widespread influence of Crofts' (1937,

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Abbreviations: LRM = larval retractor muscle, ACC = accessory larval retractor muscle.

1955) descriptions of shell muscles in larval archaeogastropods, which were based on paraffin sections, her results have never been confirmed using techniques permitting higher resolution. Indeed, the fact that her descriptions have not consistently coincided with observations by others (Smith, 1935; Bandel, 1982) underlines the need for in-depth study of the development of these muscles. Speculations about ancestry and evolutionary diversification of structures must be based on accurate information about the extant products of structural evolution.

The purpose of my study is not to test the theory claiming a muscle-based mechanism for generating ontogenetic torsion in archaeogastropods, but to reexamine the inventory of larval shell muscles in a haliotid and the fate of these muscles at metamorphosis. Evidence derived from light and transmission electron microscopy of sequential development stages of *Haliotis kamtschatkana* Jonas, 1845, indicates that four, rather than two, shell-attached muscles differentiate during the premorphophic phase of this species. This interpretation is summarized by the sketches in Figures 1A, C. In particular, I suggest that insufficient resolution and inadequate methods to prevent muscle contraction during fixation may have led Crofts (1937, 1955) to confuse an early developmental stage of a transient muscle projecting to the mantle, with a later differentiating pedal muscle. Observations described in this report extend those given in an earlier study on overall larval morphogenesis of *H. kamtschatkana* (Page, in press), and they indicate that the evolutionary derivation of shell muscles in haliotid gastropods should be reevaluated.

Materials and Methods

Adults of *Haliotis kamtschatkana* were collected from shallow subtidal areas around the southern tip of Vancouver Island, Canada (collection permit obtained from Canadian Department of Fisheries and Oceans), and were induced to spawn by the hydrogen peroxide method of Morse *et al.* (1976). After spawning commenced, adults were rinsed briefly in flowing seawater and transferred to aquaria containing seawater without hydrogen peroxide, where spawning continued. Collected eggs were rinsed with Millipore-filtered seawater (0.45- μ m pore size) before and after they were fertilized with a dilute sperm suspension. After larvae hatched, they were maintained in beakers containing 500 ml Millipore-filtered seawater (12° to 13°C), which was changed daily. Antibiotics (streptomycin sulfate at 50 μ g/ml and penicillin G at 60 μ g/ml; both from Sigma) were added on days 6 and 9 postfertilization. Red coralline algae (*Lithothamnion* sp.) or 0.01 mM gamma aminobutyric acid (Sigma) was used to induce metamor-

phosis of *H. kamtschatkana* larvae (indicated by loss of the ciliated velar cells), as described previously for the congeneric species, *Haliotis rufescens* (Morse *et al.*, 1979; Morse and Morse, 1984).

Larval stages were fixed at the following times after fertilization: 42, 52, 62, 72, and 96 hours; and 5, 6, 7, 9, and 12 days. In addition, young juveniles were fixed at 2, 5, and 7 days after metamorphic loss of the ciliated velar cells. All stages were anesthetized prior to fixation by an initial incubation in artificial seawater containing excess Mg^{2+} and reduced Ca^{2+} concentrations (MgSW: 225 mM NaCl, 5 mM KCl, 102 mM $MgCl_2$, 1 mM $CaCl_2$) for 15 min. Vials containing these partially anesthetized larvae were then placed on ice, and drops of a saturated solution of chlorobutanol (Eastman Kodak) in seawater were added to achieve a proportion of 1 part chlorobutanol solution : 4 parts MgSW after 5 min. The anesthetizing solution was then replaced with fixative.

Larvae were fixed at room temperature in 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.6) and 0.14 M sodium chloride for 1 hour, then stored in this fixative for up to 1 week at 10°C. Larval shells were decalcified in a 1:1 mixture of the glutaraldehyde fixative and 10% ethylenediaminetetraacetic acid (disodium salt; Sigma) for no longer than 1 hour (Bonar and Hadfield, 1974). Specimens were then rinsed three times in freshly prepared 2.5% sodium bicarbonate (pH 7.2) and post-fixed in 2% osmium tetroxide in 1.25% sodium bicarbonate buffer. Dehydration was accomplished in a graded ethanol series and specimens were embedded in Epon with propylene oxide as the transitional medium.

Histological sections (thickness 0.75 to 1 μ m) were cut with glass knives and stained with a mixture of azure II and methylene blue in borax (Richardson *et al.* 1960). Ultrathin sections were cut with a Diatome diamond knife, stained with aqueous 2% uranyl acetate (60 min) and 0.2% lead citrate (6 to 9 min), and photographed with an Hitachi 7000 transmission electron microscope.

Computer-generated reconstructions of sectioned larvae were made with Jandel's PC3D software. Profiles of the body wall (excluding the periostracum covering the decalcified shell) and selected muscles were traced from photomicrographs of sections by using a Numonics digitizing tablet. Sections of 1- μ m thickness were traced at intervals of 5 μ m, although body wall profiles in the reconstructions that also show underlying muscles are displayed at intervals of 10 μ m. The method for aligning photomicrographs of sequential sections has been described by Page (in press).

Results

Larvae of *Haliotis kamtschatkana* hatch from the egg investments at about 30 hours postfertilization (12° to

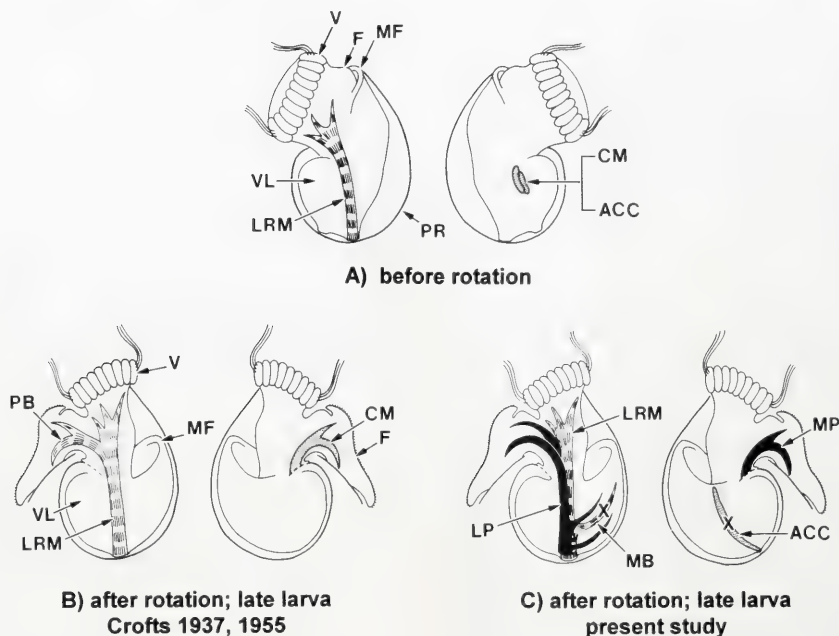


Figure 1. Two interpretations of the larval shell muscles of haliotid gastropods; sketches of muscles are simplified for clarity. (A) Right and left lateral views of a larva before rotation of the cephalopodium relative to the protoconch, showing the larval retractor muscle (LRM) and the muscle identified by Crofts (1937, 1955) as the future columellar muscle (CM) in *Haliotis tuberculata*; the latter muscle is identified as the accessory larval retractor muscle (ACC) in the present study on *H. kamtschatkana*. (B) Crofts' (1937, 1955) interpretation of the shell muscles in a late stage larva of *H. tuberculata* (shown in left and right lateral views); the LRM acquires a pedal branch (PB) and the columellar muscle (CM) projects into the foot. (C) My interpretation of the shell muscles in late stage larva of *H. kamtschatkana* (shown in left and right lateral views); the left and medial pedal muscles (LP, MP) have differentiated, but the ACC and the mantle branch (MB) of the LRM degenerate prior to metamorphosis. Other abbreviations: F = foot; MF = mantle fold; PR = protoconch; V = velum; VL = visceral lobe consisting of differentiating midgut cells.

13°C). At this time, the ovoid larval body has an encircling band of long, compound cilia (velar cilia) that powers swimming, but the foot is not yet recognizable and the protoconch (premetamorphic shell) is merely a small disc. Larvae then undergo rapid and extensive changes in both shape and cellular complexity so as to achieve metamorphic competence by 11 to 12 days postfertilization. They are able to crawl on the foot by 9 days. Major regions of larval anatomy, as referred to in the following text, are labeled on the sketches in Figure 1.

Posthatching development is accompanied by sequential differentiation and subsequent remodeling of four shell-attached muscles. Two of these, which I call the larval retractor muscle (LRM) and the accessory larval retractor muscle (ACC), begin to acquire myofilaments be-

fore rotation of the cephalopodium relative to the protoconch and visceral lobe (Fig. 1A), a morphogenetic movement that has been called *ontogenetic torsion*. However, the ACC and the mantle branch of the LRM degenerate prior to metamorphic competence. The other two shell muscles, which I call the left pedal muscle and the medial pedal muscle, differentiate after cephalopodial rotation (Fig. 1C).

Shell muscles can be unambiguously identified by their ultrastructurally distinctive attachment plaque, which is formed by specialized cells of the perivisceral epithelium (Figs. 2 and 3). As shown in Figure 3, the specialized cells are traversed by intracellular filament bundles arising from stubby apical microvilli embedded in the fibrillar matrix of the decalcified protoconch. Mem-

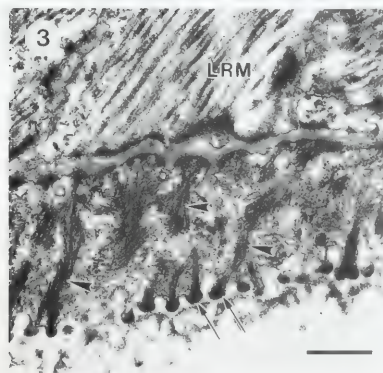
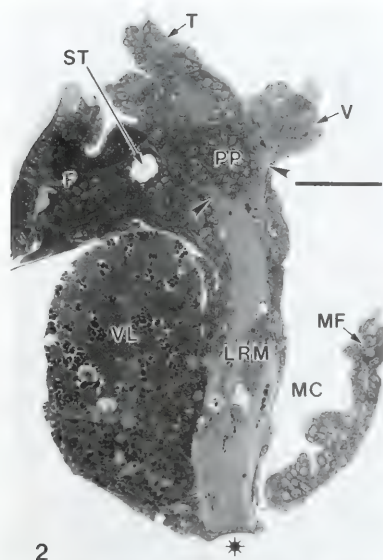


Figure 2. Parasagittal section through a larva of *Haliotis kamtschatkana* at 9 days after fertilization, showing the larval retractor muscle (LRM) projecting anteriorly from its shell attachment plaque (asterisk); distal portions of this muscle (arrowheads) extend into the velum (V) and along the ventral margin of the left pharyngeal pouch (PP). Other abbreviations: F = foot; MC = mantle cavity; MF = mantle fold; ST = statocyst; T = cephalic tentacle; VL = visceral lobe. Scale, 50 μ m.

Figure 3. Transmission electron micrograph of a portion of the larval retractor muscle attachment plaque, which consists of specialized perivisceral epithelial cells with intracellular filament bundles (arrowheads) and stubby microvilli (arrows) embedded in the fibrillar matrix of the protoconch (decalcified to allow sectioning). Scale, 0.5 μ m.

branes of both the muscle and adjacent epithelial cells have adherens-like junctional specializations that impinge on a thin layer of extracellular matrix lying between these two cell types. Attachment plaques having this morphology have been described previously for both larval and adult gastropods (Bonar, 1978; Tompa and Watabe, 1976).

Larvae also acquire musculature associated with the body wall and differentiating gut. The former consists of a complex network of intrinsic, subepidermal fibers that are particularly abundant within the foot of older larvae. The distal ends of shell muscles connect extensively with intrinsic musculature of the foot and velum. In this report, I do not describe either the gut or intrinsic body wall muscles in detail.

Larval retractor muscle (LRM) and accessory larval retractor muscle (ACC)

The images in Figure 4 are computer-generated reconstructions of transversely sectioned larvae at three stages of development: 52 hours, 5 days, and 9 days postfertilization. The reconstructions show trajectories and developmental changes of the LRM and the ACC, which are described in the following text along with micrographs of selected features.

Before cephalopodial rotation

The protoconch of *H. kamtschatkana* grows rapidly during early development, reaching final size about 52 hours after fertilization. At this stage (Fig. 5), the morphogenetic movement of cephalopodial rotation has either begun or is about to begin. Differentiating myocytes of the LRM and ACC are recognizable in these young larvae (Figs. 6 and 7) because myofilaments can be resolved within the cytoplasm (Fig. 8). Nevertheless, prior to cephalopodial rotation, most of the sarcoplasm is occupied by large yolk inclusions (Fig. 7), and myofilaments are restricted to the extreme distal ends and to a narrow cortical zone beneath the sarcolemma (Fig. 8).

Sections through the mid-level of the visceral lobe at 52 hours show profiles of the ACC and the LRM extending along opposite sides of this yolk mass of differentiating midgut cells (Fig. 6). The LRM consists of eight myocytes, whereas the ACC consists of only two (Fig. 7). No myocytes are added to either of these two muscles during subsequent development.

At 52 hours, two myocytes of the LRM are noticeably larger than the other six, and these two alone make direct contact with shell field epithelial cells that will eventually form the attachment plaque onto the shell. The basal ends of the other six LRM myocytes reach toward the bottom of the shell along the two that establish initial contact with shell field epithelium. In prerotational lar-

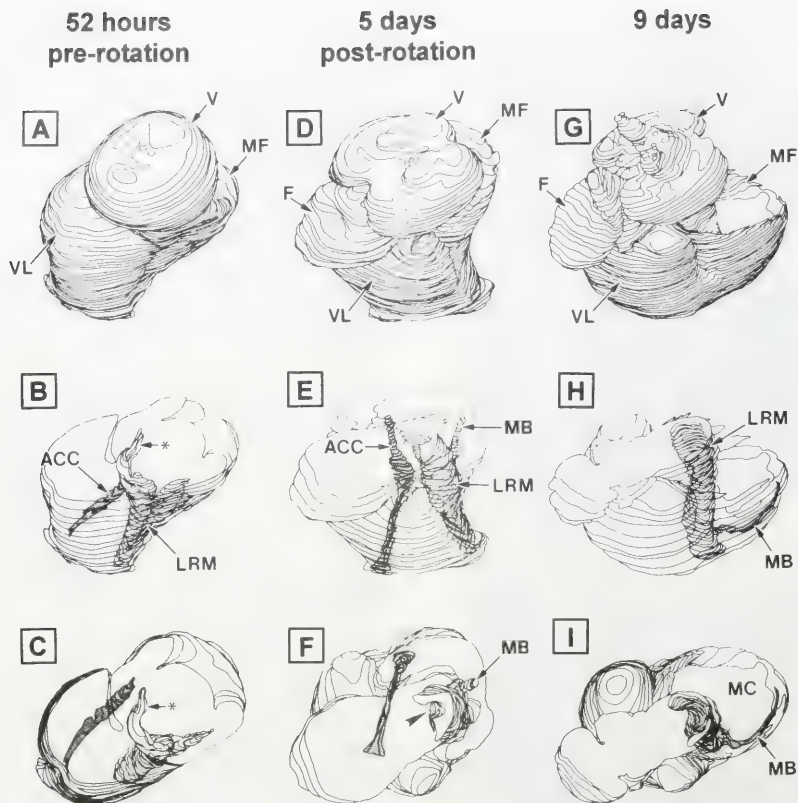


Figure 4. Computer-assisted reconstructions of transversely sectioned larvae at three stages of development (protoconch not included). The upper row shows reconstructions of whole larvae (all viewed from the definitive left lateral side of the visceral lobe) at postfertilization times of 52 hours (A), 5 days (D), and 9 days (G). The middle row (B, E, H) shows the same three stages with the anterior portion omitted and with profiles of the larval retractor muscle (LRM) and accessory larval retractor muscle (ACC) superimposed on the body wall profiles. The lower row (C, F, I) shows the same reconstructions with superimposed muscle profiles, but viewed from the anterior end of the larvae. Asterisk in (B) and (C) indicates the oblique tract of the LRM at 52 hours; arrowhead in (F) indicates the U-shaped excavation in the LRM where it partially surrounds the esophagus. The ACC is stippled in B, C, E, and F; the mantle branch (MB) of the LRM is stippled in H and I. Other abbreviations: F = foot; MC = mantle cavity; MF = mantle fold; V = velum; VL = visceral lobe.

vae, the attachment plaque of the LRM has not acquired the ultrastructural characteristics of its fully differentiated state, as shown in Figure 2, but whole mounts of live larvae suggest that epithelial cells of the future LRM attachment plaque are nevertheless fastened to the inner wall of the shell at this stage (Fig. 5). The attachment site is located at the bottom of the protoconch, but is offset toward one side. The offset is right-sided with respect to the prerotated cephalopodium, but becomes left-sided

with respect to the postrotated cephalopodium (Fig. 4: compare A–C with D–F).

The two largest myocytes of the LRM at 52 hours have different distal trajectories. One continues anteriorly along the right side of the foregut and is accompanied by four smaller LRM myocytes. The other of the two largest myocytes bends abruptly to the left, traveling dorsally over the foregut to the opposite side of the larval body. Two additional LRM myocytes show this same oblique

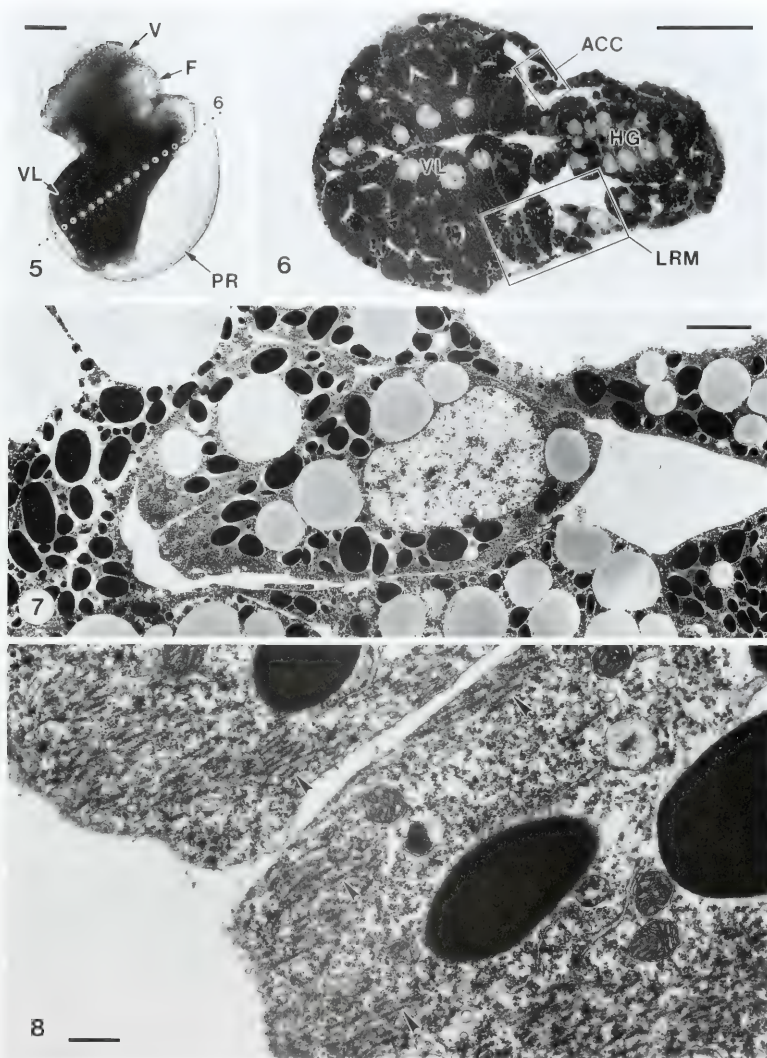


Figure 5-8. Prerotational larvae (52 hours after fertilization).

Figure 5. Lateral view of a live larva; note that the foot rudiment (F) and visceral lobe (VL) are on opposite sides of the body. Dotted line indicates approximate plane of section for Fig. 6. PR = protoconch; V = velum. Scale, 50 μ m.

Figure 6. Transverse section through the mid-level of the visceral lobe (VL), showing profiles of the larval retractor muscle (LRM) and the smaller accessory larval retractor muscle (ACC). HG = rudimentary hindgut. Scale, 50 μ m.

Figure 7. Transmission electron micrograph (TEM) of the two myocytes of the ACC containing many yolk platelets having two different electron densities. Plane of section is similar to that shown in Fig. 6, but was cut from a different larva. Scale, 5 μ m.

Figure 8. TEM showing differentiating myofilaments (arrowheads) within the peripheral cytoplasm of ACC myocytes. Scale, 0.5 μ m.

trajectory, which is marked by an asterisk in Fig. 4, B and C. As I have described previously (see fig. 9a, b of Page, in press), the oblique tract of the LRM in prerotational larvae inserts not only on columnar epithelium at the far left side of the foot rudiment (an insertion site described by Crofts [1937] for the oblique tract of the LRM in *Haliotis tuberculata*), but also on adjacent epithelium lining the initial mantle cavity and on shell field epithelium.

Prior to cephalopodial rotation, the distal ends of the two ACC myocytes do not project beyond the visceral lobe of the larval body (Fig. 4B). At 52 hours, the basal ends of these myocytes reach toward their definitive anchorage site on the protoconch, but a differentiated attachment plaque is not yet recognizable.

After cephalopodial rotation

Figure 9 shows a larva of *H. kamtschakana* at 5 days postfertilization, which is 2 days after the cephalopodium has rotated by 180° relative to the protoconch and visceral lobe (compare the position of the foot relative to the shell in Figs. 5 and 9). As larvae develop beyond the end of the rotation phase, myocytes of the LRM and the ACC enlarge and the proportion of myofilaments relative to yolk inclusions increases dramatically. Concurrently, myofilaments of both muscles become organized into short sarcomeres delimited by rows of Z-bodies (Fig. 10). However, the sarcomeres have a peculiar zig-zag pattern, which may be a fixation artifact imposed on cross-striated fibers that lack strong linkages between adjacent Z-bodies.

At 5 days posthatching, the basal trunks of both the LRM and the ACC can be seen clearly in whole mounted, live larvae (Fig. 9) and in tissue sections (Fig. 11). The attachment plaque of the LRM is located slightly toward the left side of the postrotational shell, whereas the attachment plaque of the ACC has a more ventro-medial location within the bowl of the protoconch.

As shown in the reconstructions in Figure 4, D–F, the trunk of the LRM ascends directly up the left side of the larva from its left-sided attachment plaque, whereas the two muscle cells of the ACC project almost horizontally from their shell attachment plaque so as reach toward the dorso-lateral right side of the visceral lobe. As a result, transverse sections through the extreme base of the visceral lobe cut the LRM in cross-section, but pass through the ACC in oblique longitudinal section (Fig. 11). After reaching the right side, the striated fibers of the ACC continue anteriorly into the right, ventro-lateral area of the mantle fold (Fig. 4, D–F; Figs. 12–15). The ACC extends much further anteriorly at 5 days (postrotational larva) than at 52 hours (prerotational larva).

Upon reaching the proximal level of the developing

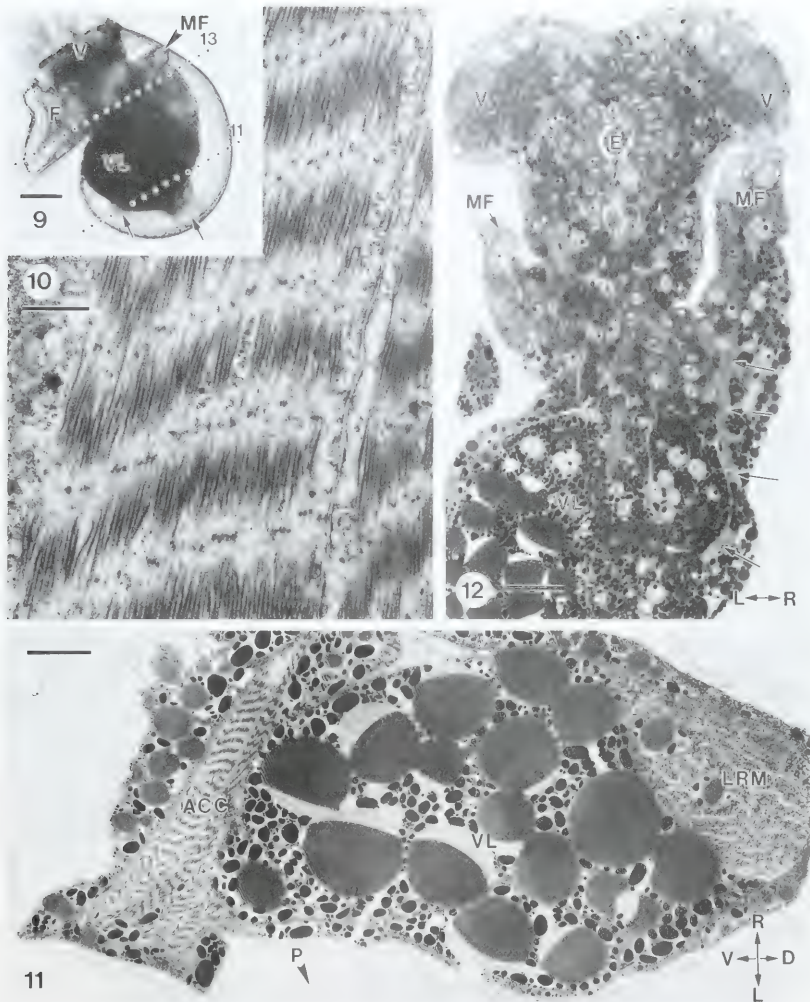
foregut, six of the eight LRM myocytes remain in close contact and collectively assume a U-shaped cross-sectional profile that invests the dorsal and lateral walls of the esophagus (Figs. 4F, 14). Eventually these six separate into left- and right-sided tracts, which insert on opposite sides of the velum and stomodeal area. The stomodeal fibers also connect with intrinsic muscle fibers extending into the foot.

The other two myocytes of the LRM branch away from the dorsal area of the main muscle bundle and extend into mantle tissue (MB in Figs. 4E, 4F, 14). However, a gradual change in shape of the mantle cavity during the period between the end of cephalopodial rotation and the onset of metamorphic competence forces a change in the disposition of the mantle branch of the LRM. For 2 full days after the cephalopodium has completed its rotation by 180 degrees relative to the protoconch, most of the initial mantle cavity is located along the right side of the larva (Fig. 13). During this initial postrotational period, the mantle branch of the LRM follows a straight anterior trajectory along the mid-dorsal border of this initial mantle cavity (Figs. 4E, 4F, 14). Subsequently, however, the right-sided mantle cavity deepens greatly and its mid-dorsal margin (including the rudiment of the initial ctenidium) pushes over to the left side, thereby creating a broad, mantle-lined cavity over the dorsal area of the body (Fig. 4, G–I; Fig. 16). As this happens, the mantle branch of the LRM is forced to diverge from the main muscle bundle at a more posterior level, and the branch becomes a curved arc that embraces the far wall of the much enlarged mantle cavity (compare Fig. 4, F and I; also Figs. 14 and 17).

The two myocytes of the ACC and the two myocytes of the mantle branch of the LRM, both of which project into the mantle fold, do not survive to the end of the obligatory larval period. In sections through larvae fixed at 9 days postfertilization, myocytes of the ACC are absent altogether or appear as a globular mass at the base of the visceral lobe. The mantle branch of the LRM is present at 9 days but not at 12 days postfertilization.

Left and medial pedal muscles

At the onset of cephalopodial rotation at 52 hours, the foot rudiment is merely a low swelling immediately beneath the stomodeum, although a small, thin operculum is present. However, dramatic enlargement of the foot begins during cephalopodial rotation and continues through the remainder of the obligatory larval period. During the initial enlargement, the foot becomes filled with a proliferating mass of cells. By 4 days postfertilization (24 hours after the end of cephalopodial rotation), occasional aggregations of myofilaments can be resolved within some of these pedal cells. Sections through pro-



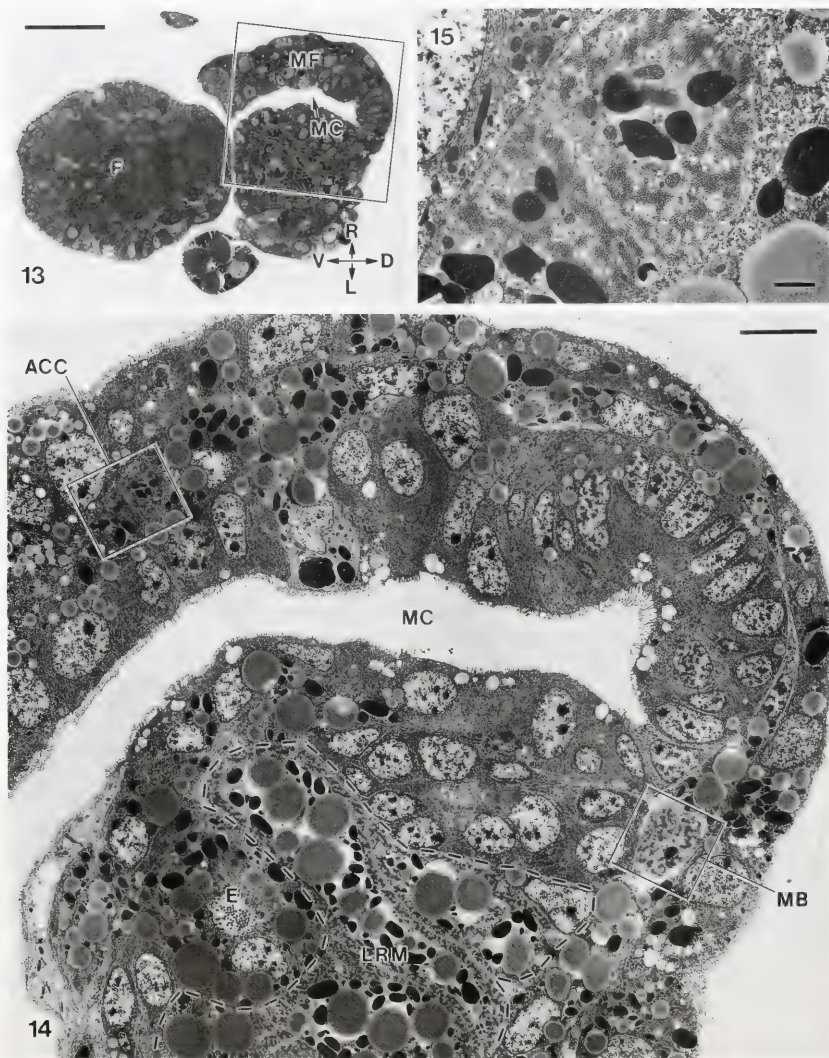
Figures 9–12. Postrotational larvae at 5 days after fertilization.

Figure 9. Lateral view of live larva; enlarged foot (F) now projects on same side as the visceral lobe (VL). Shell attachments of the larval retractor muscle (LRM) and the accessory larval retractor muscle (ACC) are indicated by arrows. Planes of section for Figs. 11 and 13 are indicated by dotted lines. MF = mantle fold; V = velum. Scale, 50 μ m.

Figure 10. Transmission electron micrograph (TEM) of striated myofilament arrangement within a myocyte of the ACC. Scale, 1 μ m.

Figure 11. TEM of a transverse section that passes through the basal trunks of both the LRM and ACC. P = periostracum of decalcified protoconch; VL = visceral lobe. Orientation axes: V = ventral; D = dorsal; R = right; L = left. Scale, 10 μ m.

Figure 12. Frontal section showing the striated myofilaments of the ACC (arrows) extending anteriorly into the ventro-lateral right side of the mantle fold (MF). E = developing esophagus; MF = mantle fold; V = velum; VL = visceral lobe. Orientation axes: L = left; R = right. Scale, 50 μ m.



Figures 13–15. Postrotational larvae at 5 days after fertilization.

Figure 13. Transverse section along the plane indicated in Fig. 9 and passing through the foot (F) and the mantle fold (MF) bordering the deep mantle cavity (MC) on the right side. Boxed area shown in Fig. 14. Orientation axes: D = dorsal; V = ventral; L = left; R = right. Scale, 50 μ m.

Figure 14. Transmission electron micrograph (TEM) of boxed area in Fig. 13 (cut from different larva); dashed lines outline a portion of the main body of the larval retractor muscle (LRM) that partially surrounds the developing esophagus (E). The section also shows the accessory larval retractor muscle (ACC) within the right-sided mantle fold (enlarged in Fig. 15) and the mantle branch (MB) of the LRM located mid-dorsally. MC = mantle cavity. Scale, 1 μ m.

Figure 15. Enlargement of the ACC from Fig. 14. Scale, 1 μ m.

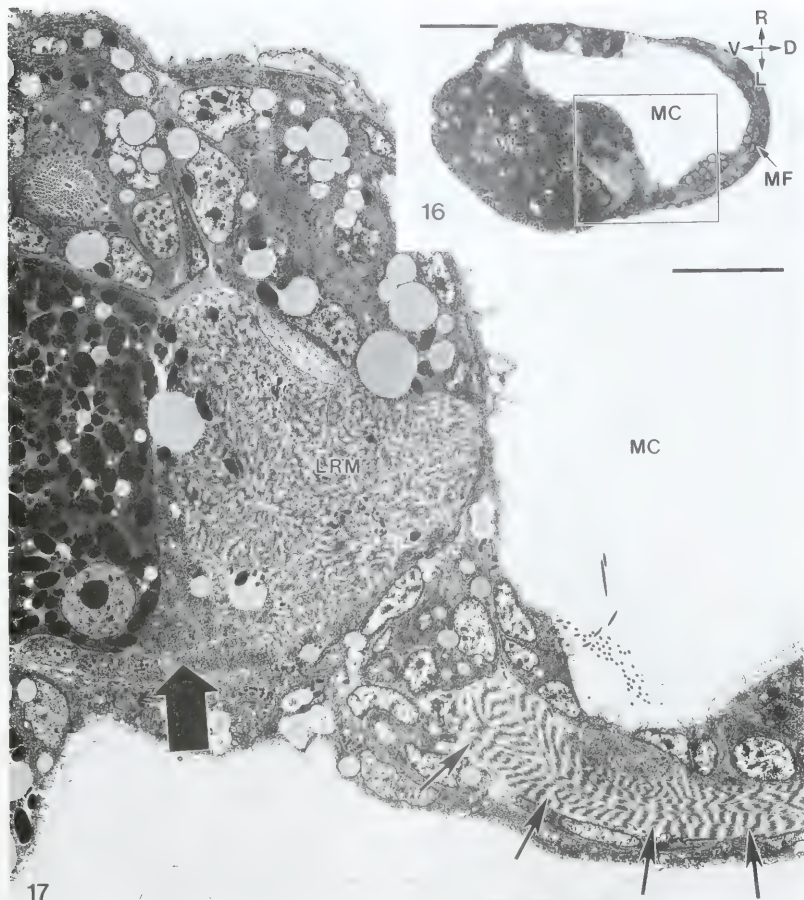


Figure 16. Transverse section of larva at 9 days after hatching, showing the enlarged mantle cavity (MC); boxed area is shown in Fig. 17. MF = mantle fold; VL = visceral lobe. Orientation axes: D = dorsal; V = ventral; L = left; R = right. Scale, 50 μ m.

Figure 17. Transmission electron micrograph (TEM) of boxed area in Fig. 16 (cut from different larva), showing the main body of the LRM and its mantle branch (slender arrows). The left pedal muscle (stout arrow) extends along the left side of the LRM and has a denser myoplasm than the LRM. Scale, 10 μ m.

gressively older larval stages suggest that the pedal myoblasts give rise to intrinsic pedal muscles and possibly to two shell-attached pedal muscles, which I call the left pedal muscle and the medial pedal muscle.

The arrangement of myofilaments within the two pedal muscles is similar, but is different from that found for the LRM and ACC. Pedal muscles have much longer

thick filaments and do not show obvious striations (Figs. 18–20).

The two pedal muscles have different shell-attachment sites and trajectories. The left pedal muscle is attached to the posterior area of the left side of the protoconch, and its trunk travels anteriorly along the ventro-lateral left side of the LRM (Fig. 17). Before entering the foot, the

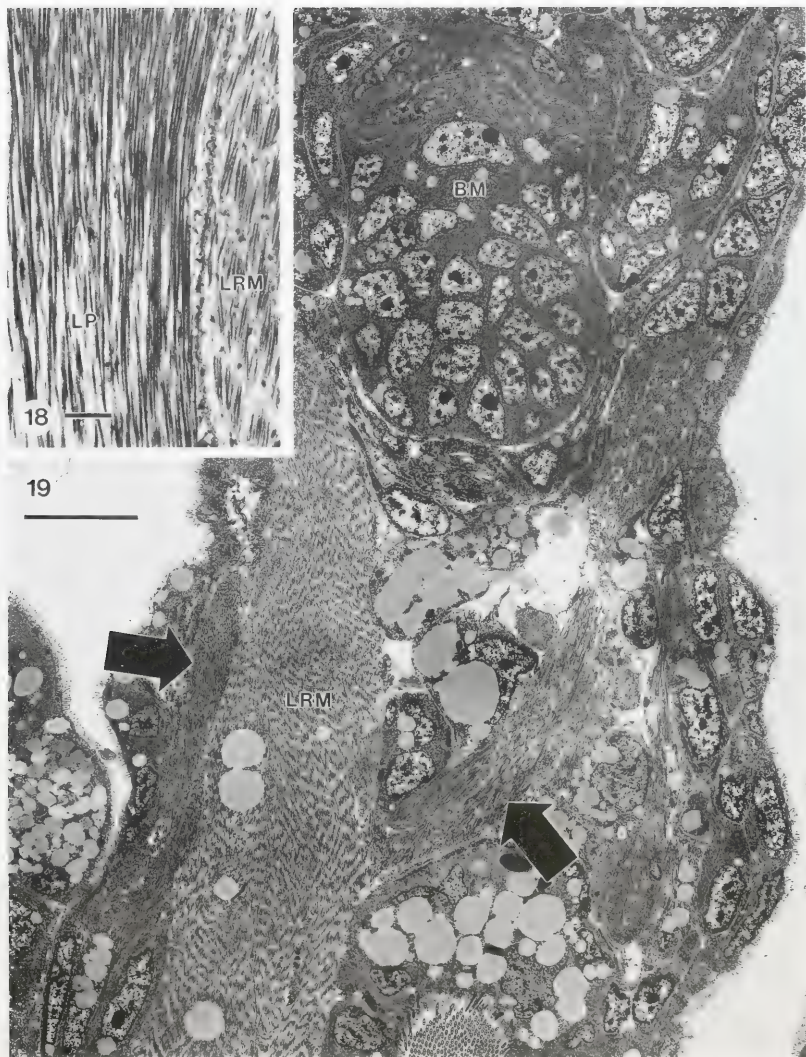


Figure 18. Transmission electron micrograph (TEM) showing different myofilament arrangements in myocytes of the left pedal muscle (LP) and the larval retractor muscle (LRM). Scale, 1 μ m.

Figure 19. TEM of a frontal section through a 12-day larva, showing the ipsilateral and contralateral distal branches (arrows) of the left pedal muscle. Unlike the LRM, the left pedal muscle does not show obvious striations. BM = buccal mass. Scale, 10 μ m.

left pedal muscle bifurcates into two distal branches (Fig. 19). One branch extends into the left (ipsilateral) side of the foot, whereas the other extends into the right (contra-

lateral) side of the foot. Therefore, sagittal sections through larvae pass obliquely through the contralateral branch of the left pedal muscle as it reaches toward the

right side of the foot (Fig. 20). Although the foot is the primary target of the left pedal muscle, this muscle also includes slender muscle slips that extend into the mantle fold.

The medial pedal muscle of late stage larvae is anchored on the external surface of a calcareous septum that is secreted along the visceral margin of the protoconch (Figs. 20 and 21); the septum is described in greater detail by Page [in press]). The muscle arches over the junction between the visceral rim of the protoconch and the operculum and extends into the central area of the foot. The various fibers of the medial pedal muscle insert on pedal epithelium underlying the operculum, on intrinsic muscle fibers associated with the crawling surface of the foot, and on the large propodial gland.

Shell muscles after metamorphosis

Sections through young juveniles at 2 to 7 days post-metamorphosis show that both pedal muscles are retained through metamorphosis and the mantle fibers of the left pedal muscle become more prominent (Fig. 22). However, the LRM, which lost the two myocytes of the mantle branch prior to metamorphosis, continues to lose myocytes after metamorphosis. Juveniles sectioned at 7 days after metamorphosis have only two LRM myocytes (Fig. 23).

By 7 days postmetamorphosis, the attachment plaque of the left pedal muscle has migrated anteriorly along the left wall of the protoconch. Also at this time, the right border of the medial pedal muscle has spread onto the right flank of the protoconch. This spreading may represent an early stage in the migration of the medial pedal muscle to the dorsal surface of the ear-shaped teleoconch, so as to eventually become the larger of the two shell muscles in adult abalone.

Discussion

Inventory of larval shell muscles: comparison to accounts by Crofts and others

Muscle morphogenesis in *Haliotis kamtschatkana* is a dynamic process that incorporates both temporal and spatial changes during the course of development. During the developmental interval that I examined, I noted differences in the time when myofilaments appeared within cells of individual muscles, and changes in details of distal insertions, migration of shell attachment plaques, and differential expression of myocyte destruction programs. However, remodeling is secondary to the fact that four shell muscles differentiate during the larval phase, and these four fall into two broad categories based on structural, functional, and developmental criteria.

One category consists of the LRM and ACC, which collectively retract the head and mantle in postrotational larvae. The striated myofibers of these two muscles begin to form before cephalopodial rotation; they differentiate from progenitor cells lying on either side of the yolk mass of differentiating midgut cells. The ACC and the mantle branch of the LRM, which both insert within the mantle fold, degenerate prior to metamorphic competence.

The second category of larval shell muscles consists of two pedal muscles. These two, together with the intrinsic musculature of the foot, may arise from progenitor cells within the foot rudiment, but this requires confirmation from cell labeling studies. The pedal muscles become recognizable only after cephalopodial rotation, and both muscles lack obvious striations. However, the medial pedal muscle is anchored on a calcareous septum secreted along the visceral rim of the shell aperture, whereas the left pedal muscle reaches posteriorly along the left side of the LRM and attaches to the postero-lateral wall of the protoconch (offset toward the left side). The medial and left pedal muscles function to pull the foot into the shell cavity during larval retraction, and their differentiation correlates with the period of rapid foot enlargement. The pedal muscles must also function to brace the shell over the foot during crawling, a behavior that emerges 9 days after fertilization (12° to 13°C) and is promoted by either red coralline algae or 0.01 mM gamma aminobutyric acid.

When Crofts (1937, 1955) did her classic studies of larval development and muscle morphogenesis in *Haliotis tuberculata* and several other archaegastropods, her analysis was restricted to observations on live larvae and paraffin sections and she was not able to prevent muscle contraction during chemical fixation (Crofts, 1937, p. 221). Improved methods, particularly electron microscopy and effective neuromuscular anesthetics, permit greater resolution of the pattern of muscle development. Although the larval shell muscles and their distal branches are theoretically resolvable by light microscopy, the high degree of structural complexity that is packed into a very small volume makes many structural details difficult to identify. I suggest that Crofts (1937, 1955) made some errors concerning the larval shell muscles of haliotids, which may have led to inappropriate interpretations of the ancestral homologues of these muscles. Nevertheless, because Crofts' descriptions and camera lucida sketches are detailed and meticulous within the limits of her technique, it is possible to identify the probable sources of discrepancy between her observations on *H. tuberculata* and the results reported here for *H. kamtschatkana*.

Most importantly, Crofts (1937, 1955) may have misidentified the ACC in prerotational larvae as an early

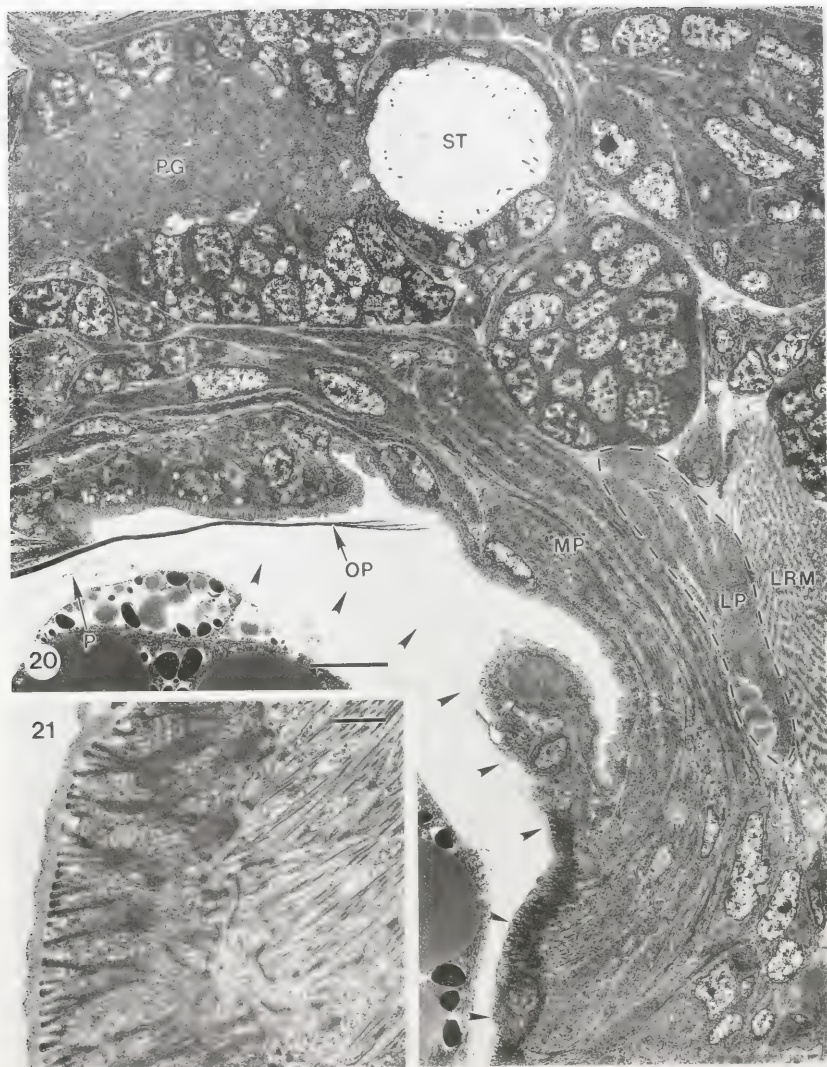


Figure 20. Transmission electron micrograph (TEM) of a longitudinal section through the base of the larval foot at 12 days after fertilization. Note the medial pedal muscle (MP) extending into the foot from an anchorage site on a calcareous shelf at the ventral apertural lip of the protoconch (arrowheads indicate decalcified matrix of septum). The section passes obliquely through the contralateral branch of the left pedal muscle (LP; outlined by dashed lines) and a small portion of the larval retractor muscle (LRM). OP = operculum; P = periostacum of decalcified protoconch; PG = right pedal ganglion; ST = right statocyst. Scale, 10 μ m.

Figure 21. TEM showing enlarged view of the attachment plaque of the medial pedal muscle. Scale, 1 μ m.

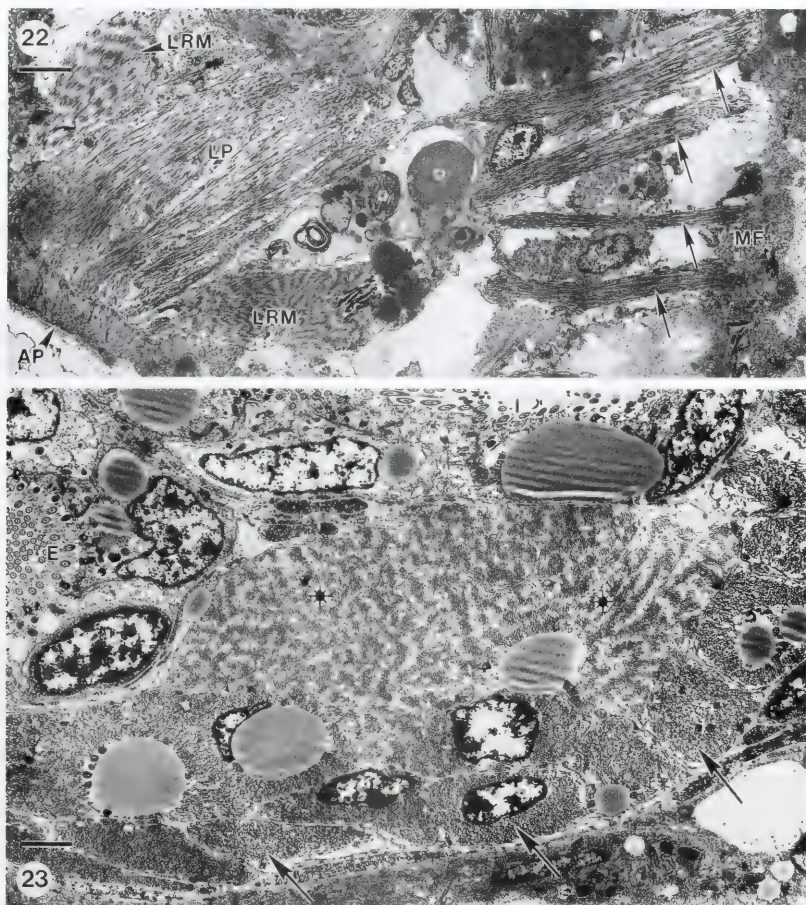


Figure 22. Transmission electron micrograph (TEM) of a sagittal section through the extreme left side of a postlarva at 2 days after velum loss; the section passes through the left side of the mantle fold (MF) and the shell-attached trunk of the left pedal muscle (LP). Digit-like muscle processes (arrows) extend from the LP into the mantle fold; asterisks indicate degenerating myocytes. AP = shell-muscle attachment plaque; LRM = myocytes of the larval retractor muscle. Scale, 5 μ m.

Figure 23. TEM of a transverse section through the LRM and left pedal muscle in a juvenile at 7 days after metamorphic loss of the velar cilia; the section passes through the left pedal muscle (arrows) where the contralateral branch is diverging from the ipsilateral branch. Only two LRM myocytes (asterisks) remain at this stage. E = esophagus; I = intestine. Scale, 2 μ m.

stage in the morphogenesis of the medial pedal muscle. She used the terms *columellar muscle* or *post-torsional right shell muscle* for both. Crofts stated: "Early in the second day [*i.e.*, before cephalopodial rotation] there are invariably two spindle-shaped cells derived from the left

mesoderm band. They are about one-third the length of the muscle cells of the right side and have no shell attachment" (Crofts, 1937, p. 230). She refers to a camera lucida sketch (fig. 25 of Crofts, 1937) of a transverse section in which the two mesoderm cells are identified as the ru-

diment of the columellar muscle. Her sketch is comparable to the section shown in my Figure 6, which also shows two myoblasts on the left side of the developing midgut. However, in *H. kamtschatkana*, these two are differentiating myocytes of the ACC, which is an entirely different muscle from the later-differentiating columellar muscle (= medial pedal muscle). The ACC of *H. kamtschatkana* soon acquires an attachment to the bottom of the protoconch and projects into the right, ventro-lateral area of the mantle fold. Indeed, the shell-attached trunks of both the ACC and the LRM can be seen for several days after larvae complete cephalopodial rotation (Fig. 9). However, Crofts (1995, p. 729) vigorously disputed a previous report on larvae of *Patella vulgata* by Smith (1935), in which he described two distinct muscles attached to separate sites along the bowl of the protoconch. However, Bandel (1982) has also reported two muscles attached to the bottom of the protoconch in early developmental stages of several trochacean archaeogastropods. One of these is clearly comparable to the LRM of haliotid larvae; the other is somewhat similar to the ACC, except that Bandel's (1982) text fig. 5 gives it a distal trajectory that is more like that of the mantle branch of the LRM in *H. kamtschatkana*.

In her 1937 paper (p. 238) Crofts states that the LRM of older larvae of *H. tuberculata* "acquires additional spindle-like fibres extending into the left side of the foot." This description may correspond to what I have identified as the distal, ipsilateral tract of the left pedal muscle in *H. kamtschatkana*. The contralateral tract, which extends into the right side of the foot, is difficult to resolve in light microscopical sections because it branches from the muscle trunk in a very structurally congested area of the larval body. Crofts interpreted these "additional fibres" as merely an elaboration of the LRM, but I suggest that they should be identified as a distinct shell muscle. Although both the left pedal muscle and the LRM attach to the shell along the left side of the bottom of the protoconch, the two muscles are different in all other respects that I could identify, including (1) temporal pattern of myofibrillogenesis, (2) primary insertion target, and (3) type of myofibril arrangement.

In summary, I suggest that Crofts (1937, 1955) may have overlooked the existence of what are actually two categories of shell muscles in haliotid larvae. Specifically, she may have misidentified the ACC as an early stage in the development of the medial pedal muscle (columellar muscle) and she may not have distinguished the left pedal muscle as separate from the LRM (Fig. 1). My suggestion could be falsified by an ultrastructural reanalysis of shell muscle development in *H. tuberculata*. It is possible that muscle development in *H. tuberculata* and *H. kamtschatkana* show real and profound differences.

Comparisons to larval shell muscles in other gastropods

Ultrastructural information on the shell muscles of other gastropod larvae is available only for members of the Nudibranchia (Bonar and Hadfield, 1974; Page, 1995). These studies reveal a common groundplan for nudibranch larval shell muscles that is similar to the pattern seen for *H. kamtschatkana* larvae. The two pedal muscles and the LRM in *H. kamtschatkana* have similar counterparts in nudibranch larvae, and in each case the LRM differentiates before the pedal muscles. However, nudibranch larvae appear to lack the ACC. Nevertheless, the nudibranch LRM incorporates a tract that projects to the right, ventro-lateral part of the right mantle fold, which is the target of the ACC in *H. kamtschatkana*. Another notable difference between the shell muscles in *H. kamtschatkana* larvae and those of nudibranch larvae is the lack of a mid-ventral stomodeal tract in the LRM of *H. kamtschatkana*.

Existing studies on caenogastropod larvae consistently describe only one shell-attached muscle (e.g., Werner, 1955; D'Asaro, 1965, 1966, 1969; Fioroni, 1966; Fretter, 1969, 1972; Fretter and Graham, 1962; Thiriot-Quievreux, 1969, 1974). This muscle initially attaches to the left side of the protoconch, but later transfers to the columella of the growing larval shell. The single shell muscle is reported to have distal branches inserting on the velum, distal foregut structures, mantle fold, and foot.

Ancestral homologues of haliotid larval shell muscles

I have categorized larval shell muscles in *H. kamtschatkana* according to characteristics of structure, function, and spatial and temporal patterns of myofibrillogenesis. These criteria delineate two categories of larval shell muscles: (1) early-differentiating cephalic and mantle retractors consisting of striated myofibers, and (2) later-differentiating pedal and mantle muscles consisting of myofibers that lack obvious striations. However, previous descriptions of shell muscles in larval archaeogastropods attempted to categorize larval shell muscles according to possible left-right homologues, and the search carried the *a priori* expectation that the homologues would show anatomical differences (see Haszprunar 1985, 1988). The reason can be traced to a preexisting evolutionary scenario for gastropods, as popularized by Garstang (1929), who proposed that gastropod torsion was the result of a mutation that created an asymmetry between left and right larval shell muscles. The hypothesized asymmetry was such that the cephalopodium of the larval body was made to twist relative to the visceropallium (i.e., the larval body underwent torsion). Garstang (1929) further suggested that this ancient evolutionary event has been preserved over many millennia and is manifest today in the ontogeny of extant

archaeogastropods. Garstang's theory about the evolutionary generation of torsion has had great impact on interpretations of gastropod evolution and phylogeny, although other aspects of his theory, including his functional interpretation of ontogenetic torsion, have been criticized by subsequent authors (Ghiselin, 1966; Thompson, 1967; Salvini-Plawen, 1980; Pennington and Chia, 1985).

Nevertheless, Garstang's (1929) theory for the evolution of torsion and the role of muscles in the torsional process was the conceptual backdrop for Crofts' (1937, 1955) interpretation of shell muscles in larval archaeogastropods. Accordingly, he proposed that the LRM and ACC are left-right homologues showing marked asymmetry in size, trajectory, and time of development. This interpretation is not disproved by the observations reported here. However, by suggesting that the ACC becomes the columellar muscle (posttorsional right shell muscle) in *H. tuberculata* and other archaeogastropods, Crofts established the precedent for the view that (1) haliotid larvae have only two shell muscles, (2) these are bilateral homologues that become the two postmetamorphic shell muscles, and (3) the two postmetamorphic shell muscles are therefore homologues of a single pair of dorso-ventral shell muscles in chitons (and presumably monoplacophorans). This interpretation is now shown to be suspect. In *H. kamtschatkana*, the LRM and medial pedal muscle belong to separate muscle groups. If the medial pedal muscle has a homologue in these larvae, the left pedal muscle is a more likely candidate than the LRM.

How can any hypothesis about bilateral muscle homologues and their role in torsion be tested (much less disproved) if both structural and developmental differences are barred from consideration because differences are actually specified by the evolutionary theory about torsion? One way out of circular arguments on this topic is to collect highly resolved morphological data on larval shell muscles for many different gastropod groups and for outgroups. Comparative developmental studies may allow muscle homologues to be identified in a way that is independent of any prevailing evolutionary theory about torsion.

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Dose-Response Relationships for Experimental Heterochrony in a Colonial Hydroid

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Abstract. Hydractiniid hydroids display a range of morphological variation. At one end of the spectrum, the colonies grow in a sheet-like configuration with their polyps close together and short stolons. At the other extreme, the colonies have a runner-like form in which the polyps are farther apart and connected by long stolons. These patterns exemplify the heterochronic variation found in many colonial animals and correspond to changes in the timing of the production of polyps and stolon tips relative to rates of stolon growth and colony maturation. Experimental studies of clonal replicates of a *Podocoryne carnea* colony demonstrated a dose-response relationship between these heterochronic traits and within-colony gastrovascular flow to peripheral stolons. A dose-response relationship was found whether flow was perturbed by manipulating the amount of food consumed by the colonies or by treating the colonies with 2, 4-dinitrophenol, an uncoupler of oxidative phosphorylation. In colonies in which flow was highly perturbed by either treatment, a similar rate of flow produced a similar morphological response. These data support the hypothesis of a causal relationship between flow rate and heterochronic variation. Nevertheless, flow was diminished by two clearly different mechanisms. Feeding manipulation altered flow relative to the size of the stolon by altering stolon thickness, without affecting the absolute quantity of flow. Uncoupling with dinitrophenol diminished the absolute quantity of flow, but did not affect the size of the stolon. A plausible assumption is that feeding manipulation affects the resistance of the stolon tissue to flow, or the fluid absorption of this tissue, or both; whereas uncoupling affects the amount of energy available to drive the flow. At the level of cellular metabolism, on the other hand, feeding manipulation

and uncoupling again have similar effects, triggering metabolic activation (*e.g.*, an increase in oxygen uptake and a shift in the mitochondrial redox state in the direction of oxidation). In the context of theories suggesting the metabolic control of development, a direct effect of feeding and uncoupling on colony development thus cannot be ruled out. Further, there may be an interaction between flow rate and metabolism, since gastrovascular flow distributes food throughout the colony, and since such substrate affects metabolic state. Both within-colony flow rate and metabolism may affect heterochronic variation in these hydroids, and methods appropriate to distinguish these two effects are discussed.

Introduction

Evolutionary changes in the timing of development underlie much of animal diversity (*e.g.*, Gould, 1977; Alberch *et al.*, 1979; Bonner, 1982; McKinney, 1988; Wake *et al.*, 1991; Hall, 1992). Historically, studies of heterochrony have focused on describing patterns of comparative embryology and morphology (Gould, 1977), and to a large degree this descriptive and correlational tradition still persists (see Raff and Wray, 1989). Thus, relatively little is known about the actual mechanisms that govern heterochronic variation. Are morphological and life-history heterochronies direct consequences of genetic and molecular heterochronies? Do physiological, developmental, and metabolic factors mediate heterochronic variation? Can unrelated genetic changes produce similar heterochronies by affecting the same epigenetic process? Investigations of such questions are crucial if studies of heterochrony are to progress beyond largely descriptive studies of morphology (Raff and Wray, 1989).

To address these sorts of questions, whole-organism experimental manipulations have become increasingly

common in studies of evolutionary morphology (*cf.*, Ketterson and Nolan, 1992; Sinervo *et al.*, 1992; Sinervo and Basalo, 1996), and this is particularly true in studies of the evolution of development (*e.g.*, Alberch and Gale, 1985; Stebbins and Basile, 1986; Meyer, 1987; Müller, 1991; DeSalle and Carew, 1992; Blackstone and Buss, 1992, 1993; Dudgeon and Buss, 1996). The central motivation for these latter studies is articulated by Stebbins and Basile (1986) in their definition of phyletic phenocopies: "We propose this term for changes in form or physiological response that mimic the normal form or reaction of a related phenotype, particularly one belonging to a different taxon. Using them, investigators have obtained clues to the developmental basis of evolutionary change, and occasionally to the nature and action of the genes involved." Experimental aspects of this approach can be particularly effective in the context of studies of heterochrony (*e.g.*, Meyer, 1987). Nevertheless, to definitively investigate the basis for heterochrony, such experiments should include not just gene dosage studies (*e.g.*, DeSalle and Carew, 1992), but also manipulations of physiological and developmental mechanisms against a uniform genetic background. Further, once an experimental basis for heterochrony has been demonstrated, the causal basis of such a relationship should be further elucidated by establishing the underlying biological gradient or dose-response curve (*e.g.*, Hill, 1965; Weed, 1988).

As model systems for experimental studies of the evolution of development, clonal organisms (*e.g.*, many fungi, herbaceous plants, and colonial invertebrates) are particularly appropriate. In such organisms, the growth and development of the colony are inseparable, and there is a broad chronological window in which manipulations of development are possible; moreover, genetically identical clonal replicates can be used in these experiments (Buss and Blackstone, 1991). In many clonal groups, the morphology can be idealized as comprising feeding and reproductive entities, here termed polyps, which are interconnected by vascular stolons. Runner-like forms (*cf.*, "guerrilla" of Harper, 1985) show widely spaced polyps and long stolon connections, while sheet-like forms (*cf.*, "phalanx" of Harper, 1985) show closely packed polyps with short stolon connections. These different morphological patterns correspond to changes in the timing of the production of polyps and stolon tips relative to the rates of stolon growth and colony maturation; high rates of production yield sheets, whereas low rates yield runners. Further, these distinctive morphologies correlate with a variety of life-history traits; runner-like forms tend to grow quickly, reproduce early, and disperse widely as compared to sheet-like forms (*e.g.*, Jackson, 1979; Harper, 1985). In the terminology of heterochrony, runner-like forms often exhibit progenesis (*i.e.*, precocious

sexual reproduction) and paedomorphic (*i.e.*, "child-shaped") adult morphologies.

Hydractiniid hydroids illustrate these general patterns; species of *Hydractinia* are typically sheet-like, while species of *Podocoryne* are typically runner-like (Blackstone and Buss, 1991; Blackstone, 1996). The morphological aspects of this variation derive from the higher rates of polyp and stolon tip formation, relative to rates of stolon growth and colony maturation, in *Hydractinia* compared to *Podocoryne*. This difference is particularly pronounced at the time of the formation of the stolonial mat in *Hydractinia*. In addition, the relative rates of polyp and stolon production show an inverse correlation with rates of gastrovascular fluid flow to peripheral stolon tips. Compared to colonies of *Podocoryne carnea*, mature colonies of *Hydractinia symbiolongicarpus* exhibit a low rate of flow to peripheral stolons, particularly subsequent to the formation of the stolonial mat (Blackstone and Buss, 1992; Blackstone, 1996).

Experimental studies of heterochrony in these hydroids have demonstrated that the between-species pattern can be mimicked by experimental manipulation of colonies of a single species (Blackstone and Buss, 1992, 1993). Putatively, these hydroids incur substantial energetic costs in circulating the gastrovascular fluid throughout the colony. Application of 2,4-dinitrophenol to colonies of *Podocoryne carnea* results in a condition of "loose-coupling" of oxidative phosphorylation, a decrease in the energy available for generating gastrovascular flow, and a consequent diminishing of the rate of flow to peripheral stolons. Correlated with this diminished flow are changes that parallel patterns of heterochrony; the rate of production of polyps and stolon tips increases relative to the rates of stolon growth and colony maturation, and peramorphic ("shapes beyond") forms result. Alternatively, gastrovascular flow can be diminished by increasing the number of times a colony is fed (*e.g.*, from 3 to 6 times per week), possibly because a higher rate of feeding increases either the viscosity of the gastrovascular fluid or the resistance to flow of the stolonial tissues, or both. Feeding manipulation, like treatment with uncouplers, results in changes that parallel patterns of heterochrony; again, the rate of production of polyps and stolon tips increases relative to rates of stolon growth and colony maturation (see Braverman, 1974). Although increased feeding produces a surfeit of nutrients and seems in many ways the opposite of the energy-poor state produced by uncoupling, its effect on colony physiology (*i.e.*, flow rate) is similar. In combination, the between-species data (Blackstone and Buss, 1992; Blackstone, 1996) and the experimental manipulations (Blackstone and Buss, 1992, 1993) suggest that flow rate is a principal mechanism underlying heterochronic alterations of these hydroid colonies.

Nevertheless, these interpretations are complicated by the similarities potentially induced by feeding manipulation and uncoupling treatments at the level of cellular metabolism (e.g., Chance *et al.*, 1963; Heytler, 1981; Chance, 1991). Briefly, feeding triggers metabolic activation, which has features (e.g., increased oxygen consumption, shift of the mitochondrial redox state in the direction of oxidation) that can be mimicked by uncoupling. In this latter case, oxidation of substrate is "uncoupled" from energy conversion, so metabolic activation does not lead to increased ATP formation. Treatments involving feeding manipulation and uncoupling may thus share some characteristics at the level of mitochondrial metabolism, but diverge at the level of ATP production. Further, the features of metabolic activation in experiments with uncouplers are often sensitive to the nature of the particular uncoupler used, its concentration, and other aspects of the experimental protocols.

Given these considerations, further investigations into the relationship between gastrovascular flow rate and heterochronic variation in these hydroids are needed. Here, two series of experiments are reported. The first series focused on causal criteria: if a dose-response relationship exists between flow rate and heterochronic variation, a causal link between these variables gains considerable support (e.g., Hill, 1965; Weed, 1988). Hence, two dose-response experiments were carried out with clonal replicates of the same *P. carnea* colony. The first experiment examined dose-response in feeding experiments. Although the actual experimental treatment is the number of feedings per week, gastrovascular flow rate (the hypothetical physiological dose) is considered the predictor variable; morphological development relative to the timing of the sexual (medusoid) phase of the life cycle is considered the response variable. The second experiment examined dose-response in uncoupling experiments. Again, gastrovascular flow rate (the hypothetical physiological dose) is considered the predictor variable, and morphological development relative to the timing of the sexual (medusoid) phase of the life cycle is considered the outcome variable. Both experiments support the hypothesis of a dose-response relationship between gastrovascular flow and heterochronic variation in these hydroids.

The second series of experiments directly investigated metabolic characteristics of colonies subjected to feeding manipulation and uncoupling; oxygen uptake and mitochondrial redox state were measured in clonal replicates of the same *P. carnea* colony. The results suggest some parallels between these treatments at the level of cellular metabolism, although the extent to which such features can directly affect colony development remains unexplored. However, these results complicate the interpretation of the dose-response relationships, and these com-

plications, and the methods appropriate for their resolution, are discussed. At this time, it seems possible that both colony physiology (i.e., flow rate) and metabolism may mediate the genetic aspects of heterochrony in these hydroids.

Materials and Methods

Study species

Podocoryne carnea exhibits traits typical of runner-like colonial animals (Blackstone, 1996). Colony development begins with the metamorphosis of the planula larva into a primary polyp. Runner-like stolons extend from the primary polyp. Stolons encase fluid-filled canals that are continuous with the gastrovascular cavity of the polyp. In cross section, stolons consist of a fluid-filled lumen encased by endoderm, ectoderm, and a rigid periderm. Gastrovascular fluid circulates in the lumen of the stolons and carries food and possibly other metabolites from the feeding polyp to other parts of the colony; contractions of the muscular polyp propel the gastrovascular fluid (Schierwater *et al.*, 1992). As the lumen fills and empties in response to contractions of the polyps, the endodermal and ectodermal tissue layers of the stolon expand and contract as well. The rigid periderm, however, remains fixed and sets the maximum diameter for stolon expansion. In cross section, the stolon (and lumen) is not cylindrical but more closely resembles a half ellipse with the "flat" surface adjacent to the substratum. During tissue expansion and contraction in response to flow, the lumen cross section may change shape, thereby complicating the physical biology of fluid flow (see Blackstone, 1996; Van Winkle and Blackstone, 1997).

Colony development from a primary polyp can be mimicked by surgically explanting 1–2 polyps from a colony onto a new surface. In both cases, *P. carnea* develops by lineal extension of the stolons, initiation of new stolon tips, and iteration of feeding polyps on the stolons, and forms a loose network of polyps and stolons typical of many runner-like forms. Once the available substratum is covered, *P. carnea* colonies increase polyp and stolon tip formation, producing a more closely knit network of stolons and ultimately initiating the sexual (medusoid) phase of the life cycle. When genetically identical clonal explants of the same colony are used, the development of treated colonies can be manipulated relative to control colonies, producing more sheet-like *P. carnea* and thus providing an experimental system to generally examine heterochronic variation in runners and sheets (Fig. 1).

Production of colonies and culture conditions

Colonies on hermit crab shells were collected from the Yale Peabody Museum Field Station in Connecticut.

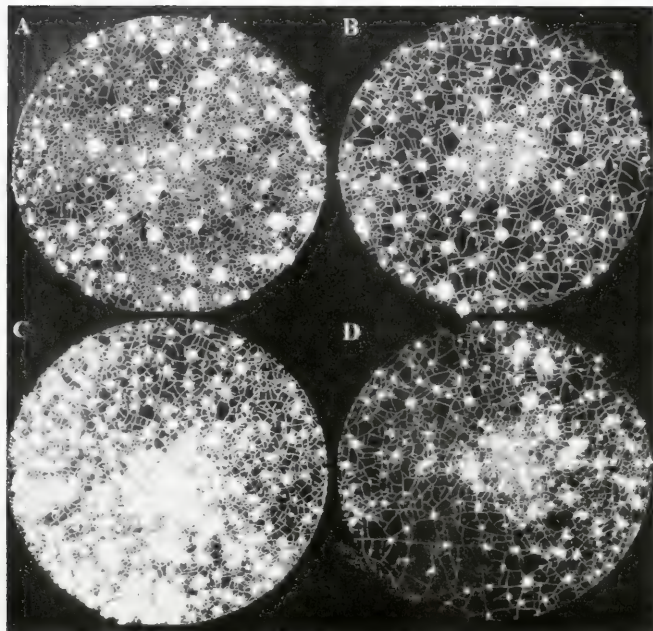


Figure 1. Background-subtracted images of several treated and control colonies of *Podocoryne carnea*: (A) treated with $30\ \mu\text{M}$ dinitrophenol; (B) dinitrophenol control; (C) fed 6 times per week; (D) feeding control. The genetically identical colonies encrust 15-mm diameter glass cover slips and were imaged at the time of the initiation of medusa production (data from these images are included in Figs. 3 and 6). In each image, polyps are bright and circular, stolons are darker and web-like, and the substratum appears black. Both increased feeding and uncoupling increase the numbers of polyps and encrusting stolons at this developmental landmark: such colonies can be considered peramorphic; *i.e.*, they exhibit "shapes beyond" the normal developmental pathways.

Clonal replicates of one *P. carnea* colony were made by explanting 1–2 polyps and their connecting stolons onto round glass cover slips. For measures of flow rate and morphology, 15-mm diameter cover slips are ideal, but for measures of oxygen uptake and redox state, 12-mm diameter cover slips are necessary. After the explants attached and began to grow, all the original explant tissue was removed from the cover slip. In all experiments except the ones measuring redox state, colonies were effectively confined to one side of the cover slips by cutting back encrusting stolons from the reverse side on a daily basis. Cover slips were suspended in floating racks and grown in 120-liter aquaria containing Reef Crystals artificial seawater (salinity = 35‰) with temperature control to $20.5^\circ \pm 0.5^\circ\text{C}$, undergravel filtration, and 50% water changes weekly. Ammonia, nitrites, and nitrates were maintained below detectable levels (Aquarium Systems test kits). With the exception of the feeding manip-

ulation experiments, colonies were fed to repletion with brine shrimp nauplii 3 days per week. Analysis has shown that, with similar culture conditions, "random" statistical effects (*e.g.*, time effects, tank effects, rack effects; see Sokal and Rohlf, 1981) are negligible (Blackstone and Buss, 1991).

Colony image analysis and morphometrics

Colonies were measured using image analysis technology (see Rohlf and Bookstein, 1990). Briefly, a high-resolution MTI CCD-72 camera attached to a macro lens was used to project an image of each colony onto a color monitor interfaced with a PC compatible microcomputer (pentium/90 MHz CPU, 32 Mb RAM) equipped with an Overlay Frame Grabber board (640×480 pixels with 12-bit depth per pixel). OPTIMAS software was used to acquire background-subtracted images of the col-

onies with illumination appropriate to produce three distinct luminance thresholds: the polyps (lightest), the stolons (intermediate), and the empty cover slip (darkest). Using these thresholds, the software identified and measured the areas of the empty (*i.e.*, unencrusted) cover slip and of the individual polyps (Blackstone and Buss, 1992). Classification macros were used to identify and exclude areas of cover slip outside the edge of the colony. The total colony area and perimeter were also measured.

Data files were analyzed using PC-SAS software. For each colony at each measurement time, the total area of polyps and the total area of empty, unencrusted cover slip enclosed within the colony were expressed as a fraction of the total area (note that the total area of stolons can be calculated as 1 minus this combined fraction). Polyp area is clearly a measure of polyp development; empty, unencrusted inner area is largely a measure of stolon development. Although polyps can shield empty, unencrusted area from observation and measurement, in practice this is a minor source of error because stolon development is generally most extensive at the base of the polyps. This is particularly true at the time of initiation of the sexual (medusoid) phase of the life cycle and subsequently. Thus, at the times in development when morphology was measured (see below), polyp area and unencrusted inner area behave as largely independent measures of two different aspects of development. Morphological differences between experimental treatments were assessed by two methods: first, using an analysis of variance with the outcome equal to the natural logarithm of the ratio of total polyp area divided by total unencrusted inner area; second, using a multivariate analysis of variance with two outcomes, the natural logarithm of the ratio of total polyp area divided by total colony area and the natural logarithm of the ratio of total unencrusted inner area divided by total colony area. (Natural logarithms were used to better meet the assumptions of parametric statistics.) Both analyses ask essentially the same questions in slightly different ways, and both give very similar results. To avoid redundancy, only the results of the MANOVA's are reported here. Additional analyses using other measures of morphology (*e.g.*, the ratio of total polyp area to mean inner area; see Blackstone and Buss, 1992, 1993) provide very similar between-treatment effects.

Video microscopic measures of peripheral gastrovascular flow

Gastrovascular flow reaches a maximum 2–8 h after feeding (Schierwater *et al.*, 1992); all of these studies were carried out 3–5 h after feeding. The colony was placed in a flow-through chamber with a #1 cover slip as its base (Warner Instruments). The temperature of the in-

flowing seawater was adjusted with a thermoelectric device to maintain a constant chamber temperature ($20.5^{\circ} \pm 0.3^{\circ}\text{C}$; chamber temperature was monitored with a YSI cuvette thermometer with a flexible probe). Colonies were viewed on an inverted light microscope (Zeiss Axiovert 135), with a $40\times$ Plan-Neo objective in differential interference contrast. Three primary stolon tips from each colony were videotaped with the MTI CCD camera for 10 minutes each.

Gastrovascular flow must be reversed in each distal, "dead-end" tip. Stolon tips fill as fluid enters; the velocity of the fluid then decreases to zero (Fig. 2). Tips then empty, and the fluid velocity again decreases to zero. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time. These width or diameter measures are taken at the base of the lumen. With the image analysis system connected to the VCR, the diameter of the stolon lumen was measured at a point $\sim 250\text{ }\mu\text{m}$ behind the tip itself. In this region of the stolon, gastrovascular fluid velocity goes to zero as the lumen diameter approaches its maximum and minimum (thus velocity itself need not be measured). Lumen diameter was measured when the stolon was full and when it was empty, for 3 consecutive, but nonoverlapping, cycles. For each cycle, the net lumen amplitude, that is, the difference between the maximum and minimum lumen diameters, was calculated. Periderm-to-periderm total stolon width (which is invariant throughout the contraction cycle) and the period (in seconds) of each cycle were also measured (Fig. 2).

Statistical analysis focused on the three measured outcomes: lumen amplitude, cycle period, and stolon width. The interpretation of these measures in terms of the volumetric rate of gastrovascular flow has been discussed in detail elsewhere (Blackstone, 1996). Briefly, these measures can be combined into a biologically meaningful measure of gastrovascular flow rate: lumen amplitude divided by cycle period and stolon width (micrometers of lumen diameter per total micrometers of stolon width per second). Biologically, this rate measure illuminates the rate of supply of food to the tissues of the stolon tip. Both this rate measure and the individual flow parameters generally meet the assumptions of parametric statistics (see Sokal and Rohlf, 1981). To compare treatments, a nested analysis of variance was used with cycles nested within stolons, stolons nested within colonies, and colonies nested within treatments. In some cases, near-significant effects that may be biologically relevant are discussed (for justification, see Rothman, 1986).

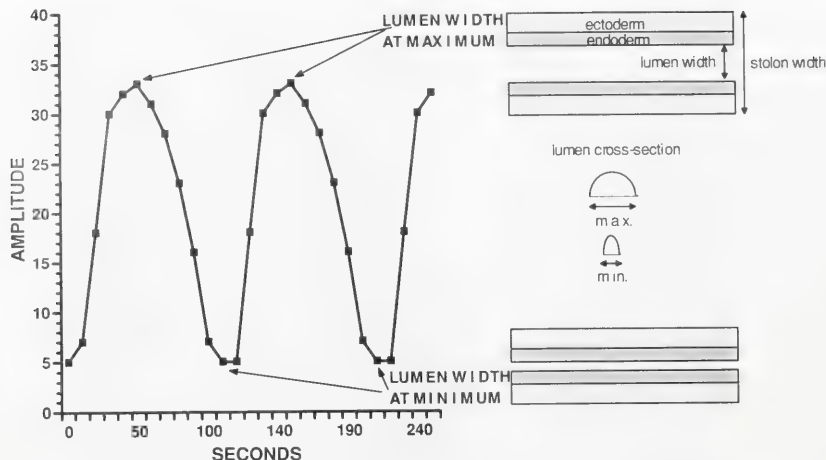


Figure 2. The contraction and expansion of the stolon lumen as it fills and empties largely in response to gastrovascular flow. Amplitude for each cycle corresponds to the diameter of the lumen. Schemata show transverse sections of the stolon tip with the lumen at maximal and minimal width. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time (modified from Blackstone, 1996).

Feeding manipulation experiments

Eighteen newly explanted clonal replicates on 15-mm cover slips were randomly divided into three groups. Each group received a different number of feedings per week ($2\times$, $3\times$ [control], and $6\times$). The morphology of each colony was measured at the time the substratum became covered, when medusa production was initiated, and when the first medusae were released. Immediately preceding the first measure, gastrovascular flow to three stolon tips per colony was measured. The initiation of medusa production was defined as the day after the first medusa buds became visible on gonozooids of the colony. The time of the first release of medusae was defined as the day after maturing medusae (*e.g.*, tentacles extended and swimming bell fully extended and contractile) were first observed on gonozooids of the colony.

Uncoupling experiments

Eighteen newly explanted clonal replicates on 15-mm cover slips were randomly divided into three groups. Each group was treated with a different concentration of 2, 4-dinitrophenol in seawater ($0\ \mu\text{M}$ [control], $15\ \mu\text{M}$, and $30\ \mu\text{M}$) for 4 h per day. Such treatment with dinitrophenol results in "loose-coupling" of oxidative phosphorylation, oxidation of NADH, and diminished ATP

formation (Blackstone and Buss, 1992, 1993). Dinitrophenol treatment was carried out in glass petri dishes containing 50 ml of the appropriate solution. Each colony was kept in a single petri dish. Dishes were arranged on three trays (one for each treatment) in an incubator (20.5°C). To randomize position and shelf effects, each colony's dish was shifted by one position each day, and trays were moved to a different shelf each day. Petri dishes were changed daily. When colonies were not being treated with dinitrophenol, they were kept in the aquaria in the normal fashion. Colony morphology and gastrovascular flow rate were measured at the same developmental landmarks as in the feeding manipulation experiments.

Dose-response relationships

In evaluating dose-response relationships between physiological parameters (gastrovascular flow rate in this case) and a morphological outcome, several considerations arise. A considerable difference is expected in the range of values between a physiological dose and a morphological response. A physiological measure is relatively instantaneous, whereas a morphological measure is cumulative. Hence, even a small physiological difference can, over time, produce a large difference in mor-

phology. The same logic suggests that this sort of dose-response relationship can best be evaluated by measures that are offset in time. In other words, the morphological effect of a physiological dose measured at one time should be assessed by morphological measures taken at later times. Given these considerations, the mean of nine measures (from three cycles of three stolons) of gastrovascular flow taken from each colony at the time the surface became covered was used to predict morphological outcomes taken subsequently (*i.e.*, at the initiation of medusa production and at the first release of medusae).

Dose-response relationships were assessed graphically and by using parametric and nonparametric measures of correlation. Gastrovascular flow rate (*i.e.*, lumen amplitude divided by cycle period and stolon width) was used as the predictor, and the ratio of total polyp area to total unencrusted inner area was used as the morphological outcome (the results were found to be insensitive to the particular morphological metric used). Both measures were natural log transformed to better meet the assumptions of the parametric correlations. However, particularly in the case of the overfeeding experiments, the assumption of a bivariate normal distribution is not well satisfied. Although all measures of correlation were similar, in this case the nonparametric correlations are most appropriate, and only Spearman's R_s are reported here (Sokal and Rohlf, 1981). Correlations between individual flow parameters (lumen amplitude, cycle period, and stolon width) and the morphological outcome were also tested.

Finally, it should be noted that the dose-response relationships for the feeding manipulation and the uncoupling experiments may not be directly comparable. In particular, previous workers have shown that confining colonies in petri dishes can lead to more sheet-like growth (Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991). In such confined cultures, soluble morphogenetic factors produced by the colonies can accumulate and enhance polyp and stolon production. The dinitrophenol experiments include this effect in all three treatments and are thus not directly comparable to the entirely aquarium-grown colonies of the feeding manipulation experiments.

Measures of oxygen uptake

Five newly explanted clonal replicates were allowed to grow on 12-mm cover slips for 2 weeks. At that point, the effect of 30 μ M dinitrophenol on the rate of oxygen consumption of each colony was measured. First, a cover slip, with its colony attached, was affixed with a drop of silicone grease to a cover slip cemented to a small magnet. This assembly was contained in a 13-mm diameter sealed glass chamber (Strathkelvin RC300) with 0.7 ml

of seawater (filtered to 0.2 μ m). Chamber temperature was held constant ($20.5^\circ \pm 0.02^\circ\text{C}$) by means of an external circulation water bath (Neslab RTE-100D), and the rate of decline in oxygen concentration over a 30-min period was measured (using a Strathkelvin 1302 electrode and 781 oxygen meter) with stirring (by slowly spinning the magnet, cover slips, and colony). Subsequently, the chamber was opened, 0.021 ml of seawater removed, 0.021 ml of 1 mM dinitrophenol solution in seawater added, the solution mixed and aerated thoroughly with a small pipette, and the chamber resealed (this procedure took ~ 7 min). The rate of decline in oxygen concentration was then measured for another 30 min. Experiments were performed 3–5 h after the subject colony was fed, as part of the normal feeding schedule. For the five colonies, the entire experiment thus took nearly 2 weeks.

After each colony was tested, a control experiment was carried out with the same five colonies and an identical protocol, except that instead of adding uncoupler, plain seawater was added prior to the second measure of the decline in oxygen concentration. Following these control experiments, feeding experiments were done, again with the same five colonies on the normal feeding schedule. In this case, a similar protocol was used, except that after the first 30-min measure of the decline in oxygen concentration, the colony was removed from the chamber, fed a small amount of brine shrimp, carefully cleaned of any shrimp or shrimp portions remaining outside the gastrovascular cavity, and returned to the chamber, which was then resealed (this procedure took ~ 20 min). The decline in oxygen concentration was then measured again. In each of these experiments, data were analyzed using a paired-comparison *t* test.

Measures of mitochondrial redox state

Spectrofluorometric assays of NAD^+/NADH provide a useful measure of mitochondrial redox state (*e.g.*, Chance and Baltscheffsky, 1958; Chance and Thorell, 1959; Chance *et al.*, 1963; Chance, 1991). These measures are carried out *in vivo*, with no apparent damage to the colonies, using a Perkin-Elmer spectrofluorometer (excitation at 366 nm and emission read near 460 nm) and acrylic cuvettes (which do not absorb or emit when excited above 300 nm). Similar experiments with a variety of clones of different hydroid species (including the *P. carnea* clone used here) have already demonstrated that uncoupling with 30 μ M dinitrophenol triggers a shift of the redox state in the direction of oxidation (Blackstone and Buss, 1992, 1993; Blackstone, *in press*). Here, the effects of feeding were investigated. Eighteen newly explanted clonal replicates were allowed to completely cover both sides of 12-mm cover slips

(~2 months). To produce an adequate signal, three colonies on cover slips were read simultaneously. Thus the effective sample size is $n = 6$. The emission of each group was measured; the colonies were then removed from the cuvette, fed a small amount of brine shrimp, carefully cleaned of any shrimp or shrimp portions remaining outside the gastrovascular cavity, and returned to the cuvette for a second measurement of emission. A control experiment was performed with the same colonies and an identical protocol, except that colonies were not fed between emission readings. Data were analyzed using paired-comparison t tests.

Results

Feeding manipulation experiments

Representative colony images can be seen in Figure 1 and on the World Wide Web (<http://www.bios.niu.edu/eande/blackstone/blackstone.html>). Feeding manipulation substantially affects colony morphology such that higher rates of feeding produce colonies with a greater area of polyps and a lesser unencrusted area within the colony (Fig. 3). At the time they completely covered the surface, colonies in each treatment are roughly similar, but there is a small between-treatment effect (MANOVA, $F = 2.93$, $df = 4, 28$, $P < 0.05$). Subsequently in colony development, the differences between treatments become profound (Fig. 3; MANOVA at the initiation of medusa development, $F = 13.2$, $df = 4, 28$, $P \leq 0.001$; MANOVA at the first release of medusae, $F = 24.7$, $df = 4, 28$, $P \leq 0.001$). At the time of covering the surface, there is also a significant between-treatment difference in flow rate (Fig. 4; using the colonies-within-treatment effect as the error term, $F = 8.9$, $df = 2, 15$, $P < 0.01$) such that a higher rate of feeding diminishes the flow. Since flow rate is a composite of three measured flow parameters (lumen amplitude, cycle period, and stolon width), it is illuminating to examine the between-treatment differences in these variables individually. Lumen amplitude and cycle period show no significant differences (Fig. 5; $F = 0.20$, $df = 2, 15$, $P > 0.8$, and $F = 1.43$, $df = 2, 15$, $P > 0.25$, respectively for each variable using the colonies-within-treatment effect as the error term). Stolon width, however, shows a dramatic between-treatment difference (Fig. 5; $F = 23.8$, $df = 2, 15$, $P \leq 0.001$, again using the colonies-within-treatment effect as the error term). A higher rate of feeding produces thicker stolons, and the between-treatment differences in flow rate derive not from differences in the amount of flow (amplitude and period), but from differences in the thickness of the stolons.

Uncoupling experiments

Representative colony images can be seen in Fig. 1 and on the World Wide Web (at the URL given in the previ-

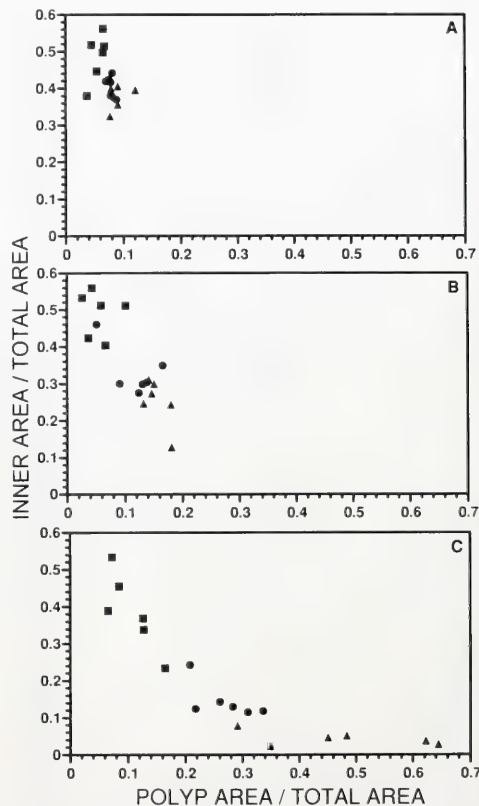


Figure 3. Bivariate scatterplots for colonies of three feeding treatments at three stages of development. Treatment: (A) 2 $\times$ per week; (B) 3 $\times$ per week; (C) 6 $\times$ per week. Stage: squares = covering the surface; circles = initiation of medusa production; triangles = first release of medusae. The y-axis is the total area of unencrusted surface enclosed within the colony; the x-axis is the total area of polyps for each colony. Both measures are adjusted for colony size.

ous section). Treatment with dinitrophenol affects colony morphology such that higher concentrations produce colonies with a lesser unencrusted area within the colony and a greater area of polyps (Fig. 6). At the time of covering the surface, colonies in each treatment are similar (MANOVA, $F = 2.0$, $df = 4, 28$, $P > 0.10$). Later in colony development, the differences between treatments become pronounced (Fig. 6; MANOVA at the initiation of medusa development, $F = 9.94$, $df = 4, 28$, $P \leq 0.001$; MANOVA at the first release of medusae, $F = 8.54$, $df = 4, 28$, $P \leq 0.001$). At the time of covering the surface, there is also a significant between-treatment

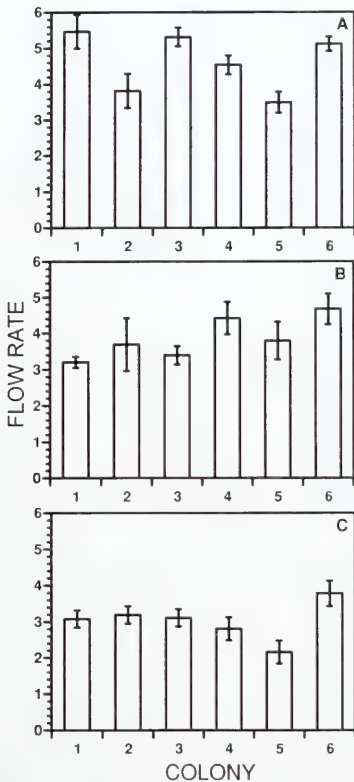


Figure 4. Means and standard errors of flow rates times 10^3 (μm of lumen expansion and contraction per total μm of stolon width per second) for the six colonies of each of three feeding treatments: (A) $2\times$ per week, (B) $3\times$ per week, (C) $6\times$ per week. Colonies of the three treatments are numbered arbitrarily.

difference in flow rate (Fig. 7; using the colonies-within-treatment effect as the error term, $F = 13.4$, $df = 2, 15$, $P < 0.001$). Treatment with dinitrophenol diminishes the flow rate. As in the feeding experiments, the between-treatment differences in these variables were examined individually. Lumen amplitude and cycle period show significant or near-significant differences (Fig. 8; $F = 7.1$, $df = 2, 15$, $P < 0.01$, and $F = 3.54$, $df = 2, 15$, $P < 0.06$, respectively for each variable using the colonies-within-treatment effect as the error term). Stolon width, however, shows no difference (Fig. 8; $F = 1.34$, $df = 2, 15$, $P > 0.25$, again using the colonies-within-treatment effect as the error term). Treatment with dinitrophenol primarily diminishes the lumen amplitude and slightly in-

creases the cycle period; the between-treatment differences in flow rate derive from differences in the amount of flow (amplitude and period), not from differences in the thickness of the stolons.

Dose-response relationships

Both experiments produced significant dose-response relationships for flow rate and morphological measures (Fig. 9). Lower flow rates produced greater polyp areas and lesser amounts of unencrusted inner areas. For the feeding manipulation experiments at the time of the initiation of medusa production (Fig. 9a), $R_S = -0.59$ ($P < 0.01$). For the feeding manipulation experiments at the time of the first release of medusae (Fig. 9b), $R_S = -0.69$ ($P < 0.001$). For the uncoupling experiments at the corresponding developmental stages (Fig. 9c and 9d), $R_S = -0.68$ ($P < 0.01$) and $R_S = -0.69$ ($P < 0.001$). There may be an effect of the small culture dishes used in the uncoupling experiments (see Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991) such that the control colonies in these experiments have greater amounts of polyp area and lesser amounts of unencrusted inner area than their counterparts in the feeding manipulation experiments. Nevertheless, for the colonies of each treatment in which flow was most perturbed (triangles on each plot), a similar flow rate produces a similar morphology.

The dose-response relationships of individual flow parameters and morphological measures correspond to the overall between-treatment effects (Figs. 5 and 8). For the feeding manipulation experiment, stolon width is highly correlated with the morphological outcome ($R_S = 0.78$, $P \leq 0.001$ and $R_S = 0.77$, $P \leq 0.001$, for the two developmental stages respectively). There are no significant correlations for lumen amplitude ($R_S = 0.16$, $P > 0.5$ and $R_S = 0.05$, $P > 0.8$, for the two developmental stages respectively) and cycle period ($R_S = 0.19$, $P > 0.45$ and $R_S = 0.34$, $P > 0.15$, for the two developmental stages respectively). For the uncoupling experiment, lumen amplitude is highly correlated with the morphological outcome ($R_S = -0.69$, $P < 0.001$ and $R_S = -0.78$, $P \leq 0.001$, for the two developmental stages respectively), and there are no significant correlations for stolon width ($R_S = 0.4$, $P > 0.1$ and $R_S = 0.07$, $P > 0.8$, for the two developmental stages respectively) and cycle period ($R_S = 0.27$, $P > 0.25$ and $R_S = 0.37$, $P > 0.1$, for the two developmental stages respectively).

Measures of oxygen uptake

Both uncoupling and feeding triggered an increase in the rate of decline of the oxygen concentration for each colony in the sealed chamber (Fig. 10). In the uncoupling experiments, for the five colonies the mean before/after

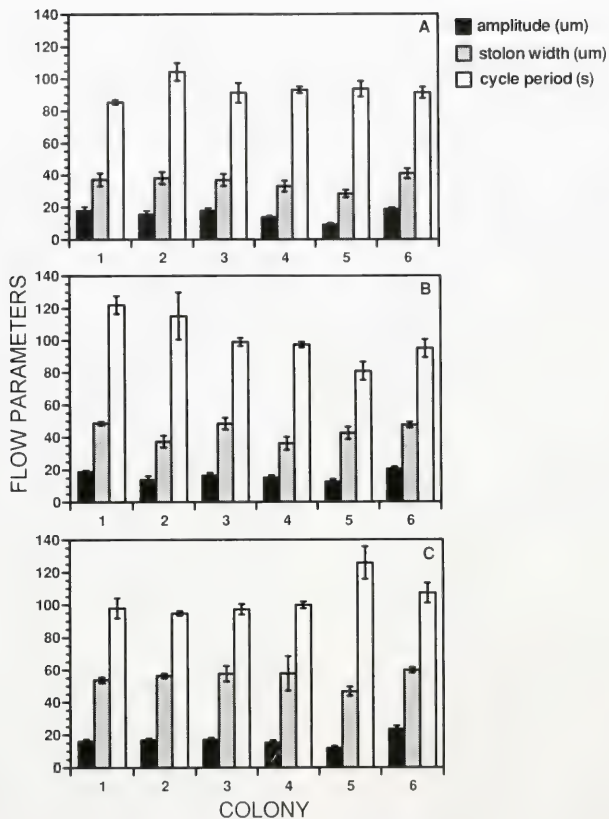


Figure 5. Means and standard errors for the three flow parameters that provide the rate measures in Figure 4 for the six colonies of each of three feeding treatments. Treatment: (A) 2× per week; (B) 3× per week; (C) 6× per week. Flow parameters: filled bars = lumen amplitude in μm ; hatched bars = stolon width in μm ; open bars = cycle period in seconds. Colonies of the three treatments are numbered arbitrarily.

difference in the slope of oxygen concentration versus time ($\pm$ standard error) = -0.008 ± 0.0017 , and this difference is significantly different from 0 (paired comparison t test, $t = -4.5$, $P < 0.01$). In the feeding experiments, for the five colonies the mean difference = -0.041 ± 0.0048 , and this difference is again significantly different from 0 (paired comparison t test, $t = -8.4$, $P < 0.001$). Note that in the latter experiments, the five colonies were about 1 month older and significantly larger than in the former. Control experiments, carried out in an identical manner with no manipulation, showed a before/after difference that was positive (mean

difference = 0.004 ± 0.001 , paired comparison t test, $t = 4.2$, $P < 0.01$). It is likely that confinement in a small chamber results in a build-up of waste products and eventually inhibits colony metabolism (see discussion in Hoegh-Guldberg and Manahan, 1995); thus the increase in oxygen consumption detected in the uncoupling and feeding experiments is probably a conservative measure of the actual effect.

Measures of mitochondrial redox state

After feeding, the peak height at 460 nm declined by an average of 42% (using units of relative intensity, the

mean before/after decline $\pm$ standard error = 4.7 ± 0.6 , paired comparison t test, $t = 7.8$, $P < 0.001$). Under the same protocol but with no feedings between the emission readings, this peak height increased by an average of less than 1% (the mean before/after increase $\pm$ standard error = 0.02 ± 0.13 , paired comparison t test, $t = 0.13$, $P > 0.9$). Since this peak corresponds to mitochondrial NADH, the data suggest that feeding caused a significant oxidation of this NADH as measured by spectrofluorometry. These results suggest an overall shift in the mitochondrial redox state in the direction of oxidation (Chance, 1991) and parallel the effect observed with

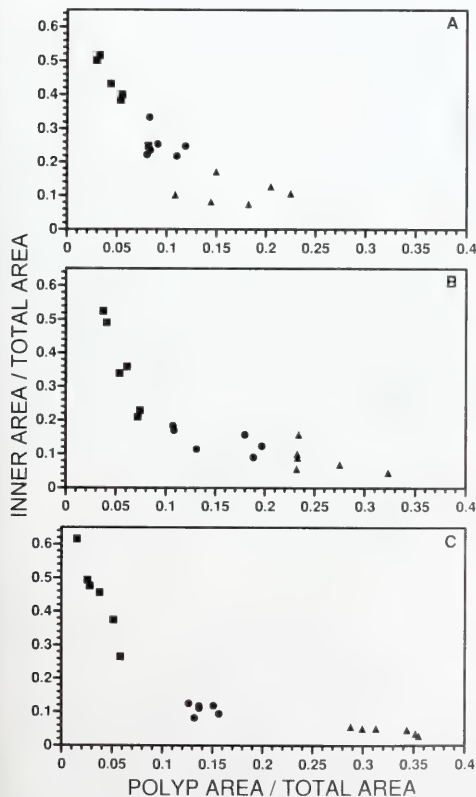


Figure 6. Bivariate scatterplots for colonies of three dinitrophenol treatments at three stages of development. Treatment: (A) 0 μ M; (B) 15 μ M; (C) 30 μ M. Stage: squares = covering the surface; circles = initiation of medusa production; triangles = first release of medusae. The y-axis is the total area of unencrusted surface enclosed within the colony; the x-axis is the total area of polyps for each colony. Both measures are adjusted for colony size.

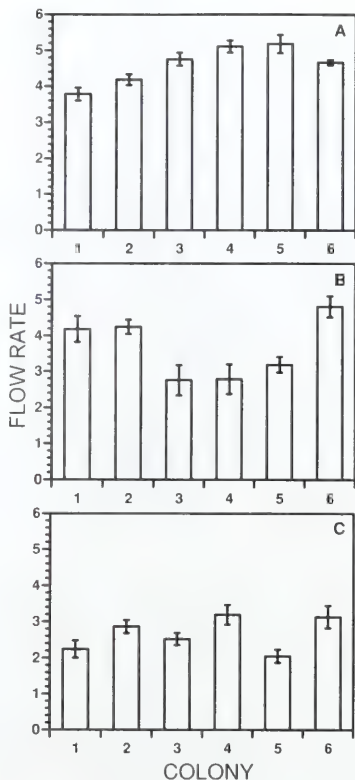


Figure 7. Means and standard errors of flow rates times 10^3 (μ m of lumen expansion and contraction per total μ m of stolon width per second) for the six colonies of each of three dinitrophenol treatments: (A) 0 μ M; (B) 15 μ M; (C) 30 μ M. Colonies of the three treatments are numbered arbitrarily.

treatments of uncouplers (Blackstone and Buss, 1992, 1993; Blackstone, in press).

Discussion

Both the feeding manipulation and the uncoupling experiments suggest that there is a dose-response relationship between the measures of gastrovascular flow to peripheral stolons and the timing of colony development in *P. carnea*. Such a biological gradient provides strong evidence for a causal relationship between these two variables (see Hill, 1965; Weed, 1988). Some differences in experimental protocols were necessary for the two experiments; in particular, treatment with dinitrophenol required confining the colonies in petri dishes. In such con-

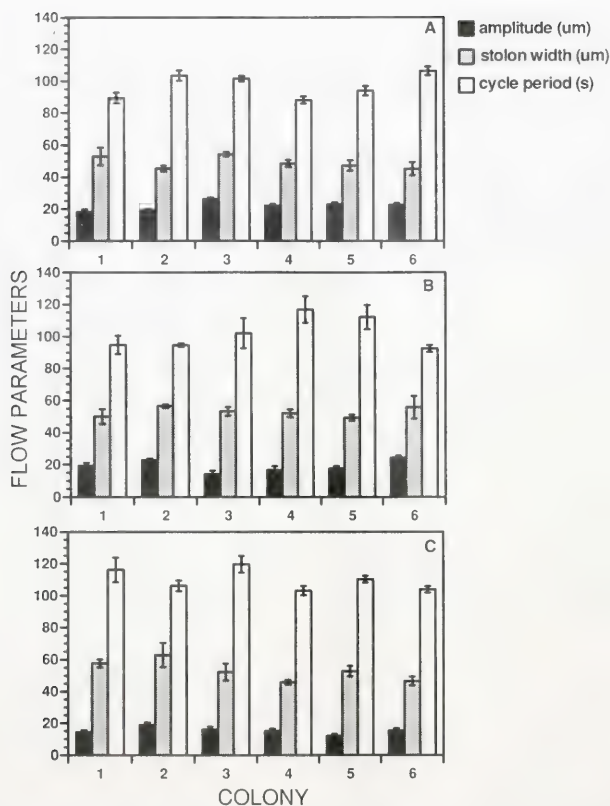


Figure 8. Means and standard errors for the three flow parameters that provide the rate measures in Figure 7 for the six colonies of each of three dinitrophenol treatments. Treatment: (A) 0 μ M; (B) 15 μ M; (C) 30 μ M. Flow parameters: filled bars = lumen amplitude in μ m; hatched bars = stolon width in μ m, open bars = cycle period in seconds. Colonies of the three treatments are numbered arbitrarily.

finer cultures, soluble morphogenetic factors produced by the colonies can accumulate and enhance polyp and stolon production (Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991). This may explain the difference between the dose-response curves in the two experiments. However, the effects of the confined cultures are most pronounced in the control colonies, whereas in the colonies in which flow was most diminished, a similar rate of flow produced a similar morphological response in both experiments. This result suggests that the rate of gastrovascular flow and the morphogenetic factors that act in confined cultures (*e.g.*, stolon-inducing factor, a 20-kDa glycoconjugate; see Lange and Müller, 1991) may interact, although the nature of this

interaction remains unclear, and alternative explanations are possible as well (see below).

Although the feeding manipulation and the uncoupling experiments both suggest that morphology is mediated by the rate of gastrovascular flow, it is clear that these treatments differ with respect to the actual parameters that underlie flow rate. Feeding manipulation strongly affects the thickness of the stolons. Underfed colonies have very narrow stolons with insubstantial tissue layers, but the actual amount of flow (as measured by lumen amplitude and cycle period) is not affected. In contrast, overfed colonies have wide stolons with thick tissue layers, and again the amount of flow is unaffected. In this way, the stolons of underfed colonies receive more

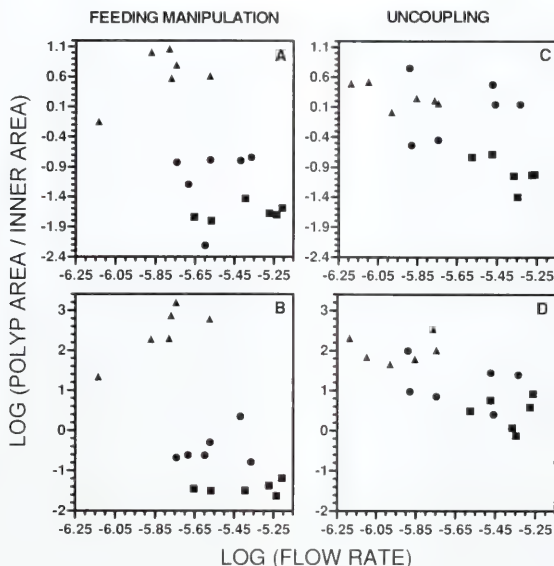


Figure 9. Dose-response relationships for feeding manipulation experiments (A, B) and uncoupling experiments (C, D). In the former, the feedings per week were manipulated (triangles = 6x, circles = 3x, and squares = 2x); in the latter, each group was treated with a different concentration of 2, 4-dinitrophenol in seawater (triangles = 30 μ M, circles = 15 μ M, and squares = 0 μ M) for 4 h per day. (A) Feeding experiment at the initiation of medusa production; (B) feeding experiment at the first release of medusae; (C) uncoupling experiment at the initiation of medusa production; (D) uncoupling experiment at the first release of medusae.

flow relative to their size than do the stolons of overfed colonies. Uncoupling, however, strongly affects the actual amount of flow, particularly as measured by the lu-

men amplitude. Stolons of treated colonies have diminished lumen amplitudes relative to controls, but the thickness of the stolon is unchanged. Thus it is in this alternative way that uncoupling diminishes the flow rate relative to the size of the stolon. A plausible conclusion is that the increased feeding and the uncoupling experiments affect flow by very different mechanisms—the former by a general thickening of the stolon, perhaps increasing the stolon's resistance to flow or its absorption of gastrovascular fluid, and the latter by diminishing the capability of the polyps to pump fluid.

On the other hand, both uncoupling and feeding produce some similarities at the level of cellular metabolism. Both treatments trigger aspects of metabolic activation as measured by oxygen uptake and mitochondrial redox state. The possible effects of metabolic state on colony development remain largely unexplored. Earlier this century, prominent hydroid biologists considered metabolic gradients to be a principal determinant of development (e.g., Child and Hyman, 1919; Child, 1941). Although these suggestions have generally been replaced by the theory of variable gene activity (e.g., Davidson, 1968), work on hydroids has continued to implicate me-

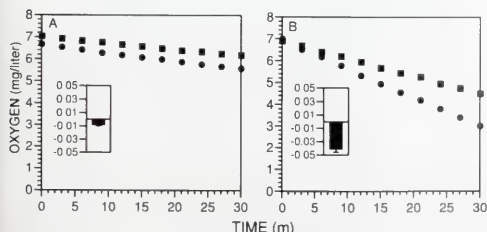


Figure 10. Rate of decline in oxygen concentration for (A) a colony before (squares) and after (circles) treatment with an uncoupler (dinitrophenol at 30 μ M) and (B) a colony before (squares) and after (circles) feeding. For five colonies, inset plots show the mean $\pm$ standard error of the before/after difference in the rate of decline in oxygen concentration, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. For both experiments, this difference in rate was significantly negative; i.e., with both treatments the oxygen uptake of the colony increased after the treatment.

tabolism in development (e.g., Newman, 1973; May and Müller, 1975). Child's theories of metabolic gradients (see Mitman and Fausto-Sterling, 1992) may yet be reconciled with the theory of variable gene activity (e.g., Nijhout, 1990; Blackstone, 1997).

In these hydroids, it is further possible that there may be an interaction between flow rate and metabolic state. Gastrovascular flow distributes food throughout the colony, and such substrate has been shown to affect mitochondrial metabolic state. The dynamics of the stolon endoderm (e.g., Schierwater *et al.*, 1992) may mediate the relationship between flow rate and food uptake, and this relationship may not be straightforward or simple. The interaction between flow rate and metabolic state is thus another possible determinant of the timing of colony development. This interaction provides another possible explanation for the somewhat different dose-response curves obtained for the uncoupling experiments as compared to the feeding manipulation experiments.

In summary, the dose-response relationships between flow rate and colony development strongly suggest that the timing of polyp and stolon tip formation in these hydroids is mediated by features of the gastrovascular system. Nevertheless, the data on metabolic activation point out the complexities inherent in analyzing experimental perturbations of this system. Since gastrovascular flow supplies substrate to the stolon tissues, any perturbation of flow alters not only the physical characteristics of the flow, but the supply of substrate as well. The latter may affect metabolic state and may act together with or separately from flow in determining the timing of polyp and stolon tip formation. In addition, the dynamics of the stolon endoderm add another layer of complexity to the effects of flow rate and substrate supply. Finally, these physiological and metabolic aspects of the stolon must ultimately interact with pattern-forming genes (e.g., Kuhn *et al.*, 1996; Cartwright, 1997) in as-yet-undetermined ways to trigger the morphogenetic events associated with polyp and stolon tip formation.

Additional experiments are clearly necessary. Crucially, these experiments must effectively distinguish between the flow rate and metabolic state *in vivo*. A variety of optical methods (e.g., Chance, 1991) and computer-automated video microscopy (Van Winkle and Blackstone, 1997) can be used to visualize redox state and flow rate simultaneously in a living stolon; then after various perturbations are applied, the rates of polyp and stolon tip development can be measured in that stolon. Such an experimental system is being developed with the expectation of better resolving the roles of flow rate and metabolic factors in the development and heterochrony of these hydroids and of other colonial animals as well.

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Homeoboxes in Sea Anemones (Cnidaria; Anthozoa): A PCR-Based Survey of *Nematostella vectensis* and *Metridium senile*

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Abstract. Homeobox genes belong to a phylogenetically widespread family of regulatory genes that play important roles in pattern formation and cell-fate specification in several model systems (e.g., *Drosophila*, mouse, and *C. elegans*). Although the evolution of many classes of homeobox genes predates the diversification of the Bilateria, comparatively little is known about homeobox genes in outgroups to the Bilateria, such as the Cnidaria. We used the polymerase chain reaction to recover 12 partial homeoboxes from 2 species of sea anemones, *Metridium senile* and *Nematostella vectensis* (phylum Cnidaria; class Anthozoa). These homeoboxes appear to represent 9 distinct, mutually paralogous homeobox genes, 5 of which belong to previously identified cnidarian homeobox classes, and 4 of which appear to represent previously unidentified classes. The evolutionary relationships between the homeodomains of sea anemones and of bilaterian animals were assessed through database searches and phylogenetic analyses. As many as 5 of the anemone homeoboxes may belong to the Hox class, which suggests that the Hox gene complement of cnidarians is larger than previously expected. Homologs of the *even-skipped* gene of *Drosophila* were also identified in both *Metridium* and *Nematostella*.

Introduction

Homeobox genes encode a family of transcription factors that are characterized by the presence of a DNA-

binding domain consisting of 60 amino acids and known as the homeodomain. Homeobox genes have been found in all metazoan phyla that have been surveyed, as well as in plants and fungi (reviewed in Bürglin, 1994). Recent reports of homeobox genes from basal metazoans, such as sponges and cnidarians, are revising our understanding of the evolution of this multigene family (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Miles and Miller, 1992; Naito *et al.*, 1993; Kruse *et al.*, 1994; Seimiya *et al.*, 1994; Degnan *et al.*, 1995; Kuhn *et al.*, 1996). Research on homeobox genes has stimulated an ongoing synthesis between the fields of evolutionary biology and developmental genetics because of the possibility that homeobox genes have played an evolutionary role in the diversification of metazoan body plans.

We identified homeobox-containing genes in two species of sea anemone, *Metridium senile* and *Nematostella vectensis*. Sea anemones belong to the Cnidaria, a phylum of tentacle-bearing, radially symmetric animals that possess nematocysts (Hyman, 1940). Substantial phylogenetic evidence places the Cnidaria near the base of the Eumetazoa, possibly as the sister group to the Bilateria. The Cnidaria share with bilaterian animals several derived characters that distinguish eumetazoans from sponges, including the possession of epithelio-muscle cells and nerve cells. However, the cnidarian body plan lacks several derived features of the Bilateria: organ-level organization, a well-differentiated mesoderm, a coelom. Furthermore, cnidarians display radial or biradial symmetry instead of bilateral symmetry. The relative simplicity of the cnidarian body plan suggests that cnidarians may possess a network of developmental regulatory genes that is relatively simple and retains more of the primitive characteristics of early eumetazoan animals than do networks found among the bilaterians.

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Abbreviations: *anth hbx*, anthozoan homeobox gene; *anthox*, anthozoan Hox class homeobox gene; *anth-eve*, anthozoan *even-skipped* class homeobox gene.

Therefore, study of cnidarians may provide unique insights into the evolution of developmental regulatory genes, such as homeobox genes. In any event, the Cnidaria are an outgroup to the Bilateria, so regardless of the degree of simplicity in their developmental regulatory networks, they will provide an important phylogenetic perspective on the evolution of development in the Metazoa.

Among extant Cnidaria, the Anthozoa (sea anemones and corals) are thought to be the sister group to a clade comprising the Cubozoa, Scyphozoa, and Hydrozoa, and it has been argued that the anthozoan body plan most resembles that of the ancestral cnidarians (Grasshoff, 1984; Schick, 1991; Bridge *et al.*, 1992; Odorico and Miller, 1997). Furthermore, the Anthozoa have a simpler life cycle than other Cnidaria, lacking the presumably derived medusoid stage that characterizes the Scyphozoa and the Hydrozoa. We have chosen to study homeobox genes in the Anthozoa that we might better understand the ancestral function of these genes in the Cnidaria and in the Eumetazoa.

Two techniques have been used to rapidly survey metazoan genomes for the presence of homeoboxes: (1) PCR (polymerase chain reaction) amplification of genomic DNA with degenerate primers corresponding to conserved regions of the homeodomain, and (2) library screening with a conserved oligonucleotide probe. More than 20 homeoboxes have been isolated from 8 cnidarian species by using these techniques (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Miles and Miller, 1992; Schummer *et al.*, 1992; Naito *et al.*, 1993; Shenk *et al.*, 1993; Aerne *et al.*, 1995). As many as five distinct homeoboxes have been recovered from individual hydrozoans, *Chlorohydra viridissima* (Schummer *et al.*, 1992) and *Eleuthera dichotoma* (Kuhn *et al.*, 1996). In a recent classification scheme (Naito *et al.*, 1993) cnidarian homeoboxes are sorted into eight mutually paralogous classes (Table I), but 18 of 20 of the cnidarian genes are from members of the class Hydrozoa. Little is known of homeobox genes in other classes, including the corals and anemones of the class Anthozoa. We have used PCR to survey the genome of two anthozoans for the presence of homeobox genes.

Materials and Methods

DNA extraction

Live anemones were obtained from William Zamer at Lake Forest College (Lake Forest, IL; *Metridium*) and Kevin Uhlinger at Bodega Bay Marine Laboratory (Bodega Bay, CA; *Nematostella*). All animals were starved for 2 weeks and rinsed several times in sterile artificial seawater prior to DNA extraction. DNA extraction was performed with proteinase K and RNase digestion fol-

lowed by several phenol/chloroform extractions (Sambrook *et al.*, 1989).

PCR amplification

Degenerate primers corresponding to highly conserved regions in helices one and three of the homeodomain were used to amplify an 82-bp fragment from genomic DNA (nucleotides 60 through 141 of the homeobox, not including the primers). The upstream primer (5'-GARYTIGARAARGARTT-3') corresponded to the amino acid sequence ELEKEF, and the downstream primer (5'-CKNCKRTTYTGRAACCA-3') corresponded to the reverse complement for the amino acid sequence WFQNR. The 5' ends of the primers were phosphorylated to facilitate cloning PCR products. Both primers were synthesized commercially (Operon Technologies, Alameda, CA).

PCR was performed in 50- μ l reactions containing 50 mM Tris (pH 8.3), 10 mM KCl, 3.5 mM MgCl₂, 200 μ M dNTPs, 0.6 Units *Taq* polymerase, 12.5 pmol of each primer, and 150 ng genomic DNA. Thermal cycling was performed in a MiniCycler (MJ Research, Watertown, MA) under the following parameters: 5 cycles (45-s each) of denaturation at 94°C, 45 s of primer annealing at 37°C, and 45 s of primer extension at 72°C, followed by 35 cycles in which the annealing temperature was raised to 42°C. Eight separate 50- μ l PCR reactions were performed for each species, and these were pooled prior to cloning to minimize the effects of stochastic variation known as PCR drift (Wagner *et al.*, 1994). Twenty-five microliters of the pooled reaction products were visualized on a 2% agarose gel stained with ethidium bromide, and the expected amplification product (114 bp) was observed. Control reactions lacking one primer or a primer pair failed to produce the expected amplification product. Control reactions lacking template produced no visible amplification products.

Cloning and sequencing

PCR products were blunted prior to cloning by using T4 DNA Polymerase (Costa and Weiner, 1994). The entire reaction was then run out on a 0.8% SeaPlaque GTG agarose gel (FMC Bioproducts, Rockland, ME) in a modified TAE buffer (0.04 M Tris-acetate, 0.2 mM EDTA). The appropriate band was excised from the gel, melted, and added directly to a ligation reaction. PCR products were cloned into pUC18 plasmid that had been cut with *Sma*I and treated with phosphatase (Kalvakolanu and Livingston, 1991). Following alkaline denaturation, double-stranded sequencing was performed on recombinant clones; the Sequenase v2.0 kit (USB, Cleveland, OH) was used with ³⁵S-labeled dATP and the M13 forward primer. Alternatively, automated sequencing was performed with dye-labeled dideoxynucleotides and

Table 1

Previously identified homeoboxes from the phylum Cnidaria

Class ^a [putative homology]	Anthozoa ^{b,c} gene/[method of isolation]	Hydrozoa ^{b,c} gene/[method of isolation]
Class I [HOM/Hox class]		<i>cnox1-Hm</i> /[A] ⁴ <i>cnox1-Ed</i> /[A] ² <i>S.tox3-Sa</i> /[A] ¹ <i>cnox1-Hv</i> /[A] ⁵ <i>cnox3-Hv</i> /[A] ⁶
Class II [Deformed of <i>Drosophila</i>]		<i>cnox2-Hv</i> /[A] ⁵ <i>cnox2-Hm</i> /[A] ⁴ <i>cnox2-Ed</i> /[A] ² <i>cnox2-Hs</i> /[A] ² <i>cnox2-Cv</i> /[B] ⁶ <i>S.tox2-Sa</i> /[A] ¹
Class III [Omi(1D), <i>Drosophila</i>]		<i>cnox3-Cv</i> /[B] ⁶ <i>cnox4-Cv</i> /[B] ⁶
Class IV [HOM/Hox class]		<i>cnox4-Hm</i> /[A] ⁴ <i>cnox1-Cv</i> /[B] ⁶
Class V [Mox1 of mouse]		<i>cnox5-Hm</i> /[A] ⁴
Class VI [?]	<i>antpC</i> -Af/[B] ¹	<i>S.tox1-Sa</i> /[A] ¹ <i>cnox1-Pc</i> /[A, B, C] ⁷
Class VII [even-skipped class]	<i>eveC</i> -Af/[B] ¹	
Class VIII [msh-like class]		<i>msh-Cv</i> /[B] ⁶

^a Naito *et al.* (1993) divided all previously identified cnidarian homeoboxes into eight classes. *cnox1-Pc* and *antpC* were not part of the original classification scheme but have been assigned to Class VI based on phylogenetic analyses (see Figs. 2 and 3).

^b All cnidarian homeobox sequences identified to date are from the Anthozoa and the Hydrozoa; the methods of isolation are indicated in brackets: [A] PCR, [B] genomic library screening, [C] cDNA library screening. No homeoboxes have yet been recovered from the classes Cubozoa or Scyphozoa. Two-letter species abbreviations are appended to the name of each gene: Af = *Acropora formosa*, Cv = *Chlorohydra viridissima*, Ed = *Eleutheria dichotoma*, Hm = *Hydra magnipapillata*, Hs = *Hydractinia symbiolongicarpus*, Hv = *Hydra vulgaris*, Pc = *Podocoryne carnea*, Sa = *Sarsia*.

^c Homeoboxes listed here are from ¹ Murtha *et al.* (1991), ² Schierwater *et al.* (1991), ³ Miles and Miller (1992), ⁴ Naito *et al.* (1993), ⁵ Shenk *et al.* (1993), ⁶ Schummer *et al.* (1992), and ⁷ Arne *et al.* (1995).

an ABI 373A sequencer. Homeobox-containing clones were sequenced in the reverse direction with the M13 reverse primer. Of the 44 clones sequenced for *Metridium*, 32 (73%) contained recognizable homeobox sequences. Of the 47 clones sequenced for *Nematostella*, 35 (74%) contained recognizable homeobox sequences.

Results and Discussion

Homeoboxes identified by the PCR-based survey

Our PCR-based survey identified 12 distinct homeoboxes in two species of sea anemone: 7 genes from *Metridium senile* and 5 from *Nematostella vectensis* (Fig. 1). Consensus sequences for each of these 12 anemone homeobox gene fragments were submitted to GenBank

(accession numbers U42726-U42737). To our knowledge, the 7 homeoboxes identified in *Metridium* are the most recovered from any single species of cnidarian. The recovery of so many distinct genes may be attributable to the initial pooling of 8 separate PCR reactions (Wagner *et al.*, 1994). The 12 anemone homeoboxes appear to represent 9 paralogous homeobox genes referred to as *anthox1*, *anthox1a*, *anthox2*, *anthox4*, *anthox5*, *anth-hbxA*, *anth-hbxB*, *anth-hbxC*, and *anth-eve*. The names were chosen to reflect putative homology between anemone homeoboxes and those from other cnidarians (see legend to Fig. 1). The PCR surveys identified overlapping but different sets of homeoboxes in *Metridium* and *Nematostella*. *Anthox1*, *anth-eve*, and *anth-hbxA* were recovered from both *Metridium* and *Nematostella*. *Anthox2*, *anthox4*, *anthox5*, and *anth-hbxB* were recov-

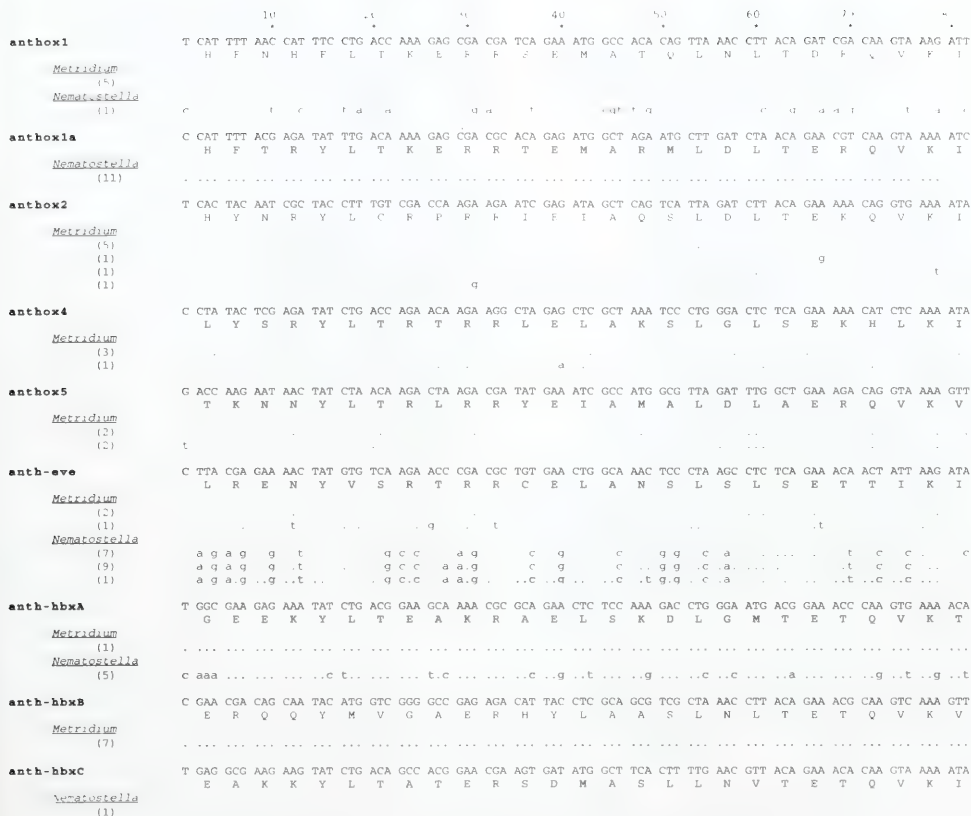


Figure 1. Nucleotide alignments of homeobox-containing clones representing 12 distinct homeobox genes, 7 from *Metridium senile* and 5 from *Nematostella vectensis*. The nucleotide alignment spans positions 61–142 of the 180-bp homeobox (amino acids 21–47 of the homeodomain). Nucleotide alignments and phylogenetic analysis suggest that these 12 homeobox fragments represent at least 9 distinct homeobox classes or cognate groups (in boldface type). The term *anthox* is used to designate anemone homeoboxes with putative homology to Hox class homeoboxes (anthozoan Hox homeobox). The term *anth-hbx* designates anemone homeoboxes lacking similarity to the Hox class. The term *anth-eve* indicates the apparent homology of this anemone homeobox fragment to the *even-skipped* homeobox of *Drosophila*. The sequences of individual clones (lowercase) are aligned to a consensus sequence (uppercase). For *anthox1*, *anthox2*, *anthox4*, *anthox5*, *anth-hbxA*, *anth-hbxB*, and *anth-eve*, the consensus sequence shown is from *Metridium*. For *anth-hbxC* and *anthox1a* the consensus sequence shown is from *Nematostella*. The inferred amino acid sequences are indicated below the consensus. The number of clones possessing a given sequence is indicated in parentheses. Identity with the consensus sequence is indicated with a period.

ered only in *Metridium*. *Anthox1a* and *anth-hbxC* were recovered only in *Nematostella*.

Comparison of homeoboxes from anemones and other cnidarians

We wanted to know if the homeoboxes we isolated are orthologous to previously identified homeoboxes from

cnidarians (Table 1; Naito *et al.*, 1993), or if they represent new classes of cnidarian homeoboxes. We used phylogenetic methods to evaluate the evolutionary relationships between representatives of the 8 putative cnidarian classes and the 12 homeoboxes recovered from *Metridium* and *Nematostella* (Saitou and Nei, 1987; Fig. 2). A neighbor-joining tree (Saitou and Nei, 1987) was constructed using the program *Phylip* (version 3.5,

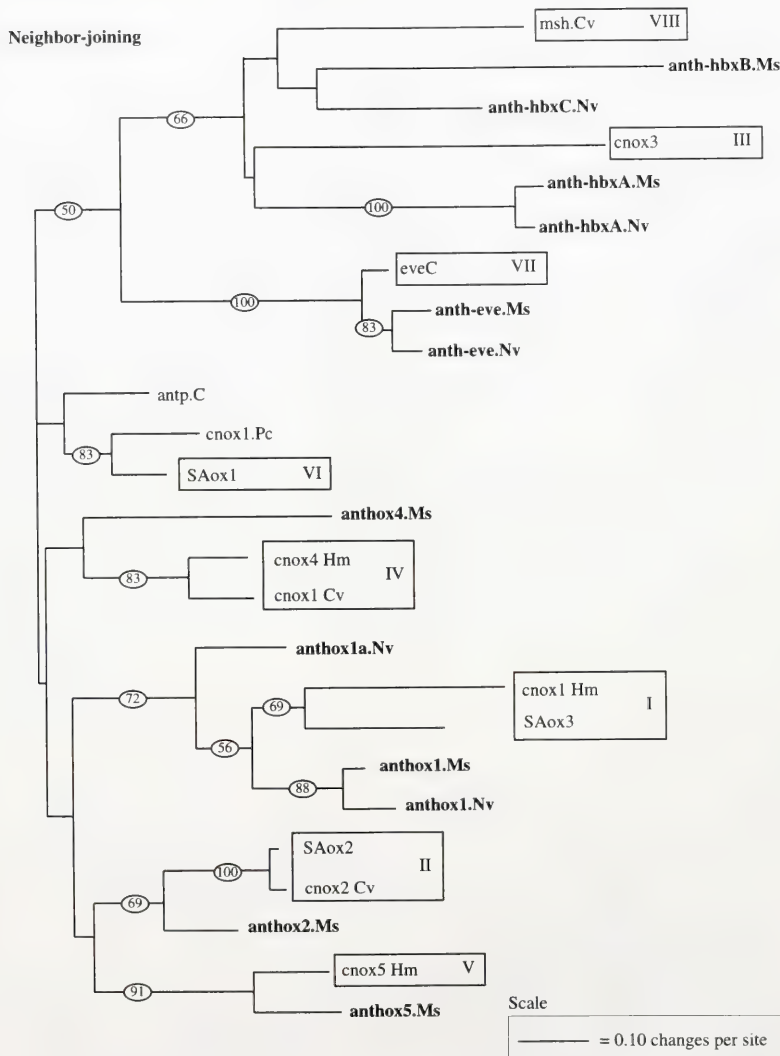


Figure 2. Phylogenetic relationships among cnidarian homeodomains were inferred using the neighbor-joining algorithm (Saitou and Nei, 1987) of PHYLIP (Phylogenetic Inference Package, version 3.5; Felsenstein, 1989). The data consisted of predicted amino acid sequences (positions 21–47; see Fig. 1) from the 12 anemone homeodomains and 13 additional cnidarian homeodomains. Anemone homeodomains are indicated in bold-face type. The cnidarian sequences were chosen to represent all 8 of the cnidarian homeobox classes proposed by Naito *et al.* (1993). Boxes enclose representative sequences from each class (roman numerals; see Table I). Sequences not enclosed in boxes (*cnox1.Pc* and *antp.C*) were not included in the classification scheme of Naito *et al.* (1993). The tree shown is unrooted. Distances between homeodomains were calculated using the Protdist program of PHYLIP and the PAM-Dayhoff amino acid substitution matrix (Dayhoff, 1978). Branch lengths in the horizontal direction are proportional to the number of expected amino acid substitutions that have occurred per site (scale at lower right). Branch lengths in the vertical direction are not significant. Circled numbers along branches indicate the percentage of trials in which a given partition between genes was found in 2000 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >40% of bootstrap replicates are indicated.

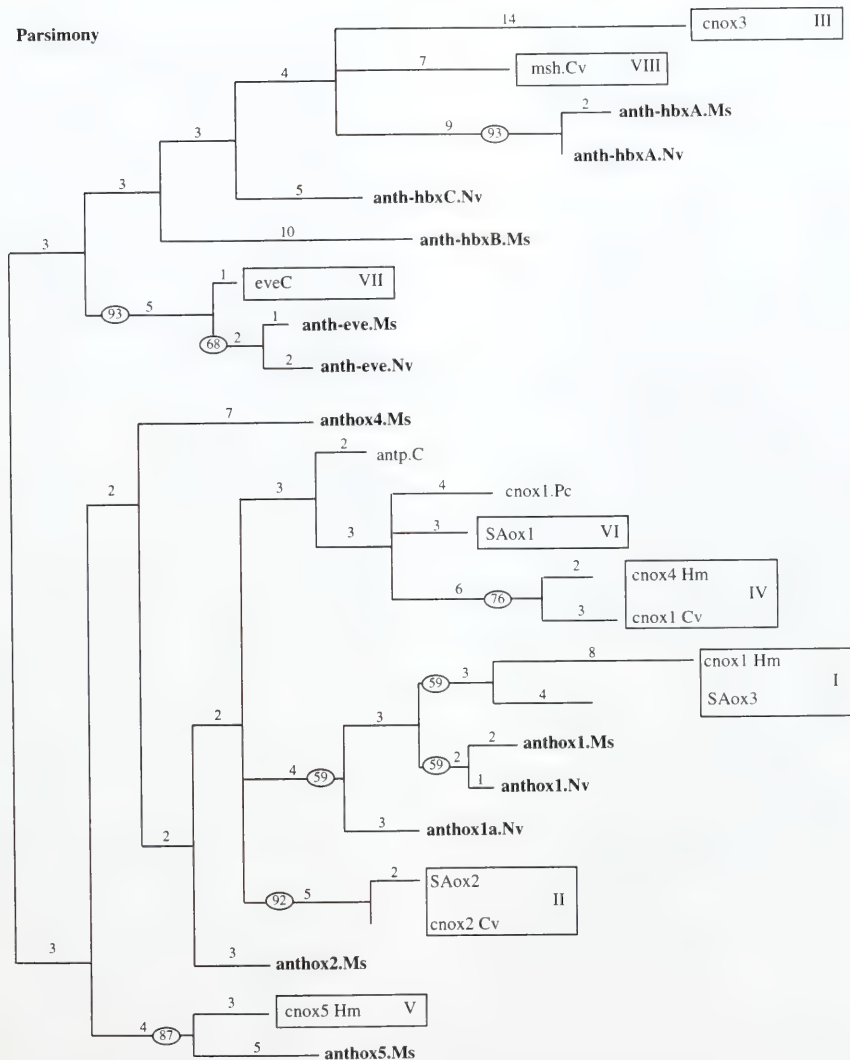


Figure 3. Phylogenetic relationships among cnidarian homeodomains were inferred by parsimony. The tree depicted is the strict consensus of 27 equally parsimonious trees identified in a heuristic search with the computer program *PAUP (Phylogenetic Analysis Using Parsimony, version 3.1, Swofford, 1991)*. Tree-bisection and reconnection branch swapping (TBR) was performed on 20 random starting trees. The tree shown is unrooted. The data consisted of predicted amino acid sequences (positions 21–47; see Fig. 1) from the 12 anemone homeodomains and 13 additional cnidarian homeodomains representing 8 distinct classes (Naito *et al.*, 1993; Table I). Anemone homeodomains are indicated in boldface type. Boxes enclose representatives from each class (roman numerals). Sequences not enclosed in boxes (*cnox1.Pc* and *antpC*) were not included in the classification scheme of Naito *et al.* (1993). Tree length is 162 steps. Consistency index (CI) = 0.691. Retention index (RI) = 0.695. Branch lengths in the horizontal direction are proportional to the number of amino acid substitutions that have occurred along each branch. The number of amino acid substitutions is specified above each branch. Branch lengths in the vertical direction are not significant. Circled numbers indicate the percentage of trials in which a given partition between genes was found in 500 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >50% of bootstrap replicates are indicated.

anthox1-Ms		HNHPLTFEERSEMATQNLNLTQVVI	identity
1. CnHv3 (<i>Hydra vulgaris</i>) [M62872]		K.....A.L.KH...SE...I.?	68%
2. Abd-A homolog (<i>Aedes aegypti</i>) [P29552]		Y.F.F.F.I.I.HA.C...E...I.?	68%
3. Hox D3 (<i>Homo sapiens</i>) [S15548]		RY.CRP...V...NL...E...I.?	62%
4. CnHv2 (<i>Hydra vulgaris</i>) [M62871]		K.....T.LSKK...SE...I.?	64%
5. Hox 3 (<i>Branchiostoma floridae</i>) [D44629]		FY.CRP...V...AM...E...I.?	64%
anthox1-Nv		HNHPLTFEERSEMPQNLNLTQVVI	
1. CnHv2 (<i>Hydra vulgaris</i>) [M62871]		F.....T.LSKK...S...I.?	68%
2. CnHv3 (<i>Hydra vulgaris</i>) [M62872]		F.....A.LAFH...S...I.?	63%
3. Abd-A homolog (<i>Aedes aegypti</i>) [P29552]		Y.F.F.F.I.I.HA.C...E...I.?	68%
4. Hox D3 (<i>Homo sapiens</i>) [S15548]		FY.CRP...V...ANL...E...I.?	63%
5. Hox 3 (<i>Branchiostoma floridae</i>) [D44629]		FY.CRP...V...AAM...E...I.?	63%
anthox1a-Nv		HFATYLTKEERTEAMRLNLTQVVI	
1. Hox A3 (<i>Mus musculus</i>) [P02831]		N...MRP...V...NL...E...I.?	70%
2. Hox D3 (<i>Homo sapiens</i>) [S15548]		N...CRP...V...NL...E...I.?	70%
3. Hox 3 (<i>Branchiostoma floridae</i>) [D44629]		N...CRP...V...A...E...I.?	71%
4. Hox B3 (<i>Homo sapiens</i>) [D37042]		N...CRP...V...NL...S...I.?	67%
5. Hox B3 (<i>Xenopus laevis</i>) [P31247]		N...CRP...V...NL...S...I.?	67%
anthox2-Ms		HYNRCLCRPRRIEIAQSLDLTEKQVKI	
1. Homeobox (<i>Carassius auratus</i>) [L09690]		K.....V...AL...R...V	78%
2. Hox A2 (<i>Notophthalmus viridescens</i>) [P31261]		F.K.....V...AL...R...V	74%
3. Hox B2 (<i>Homo sapiens</i>) [E37042]		F.K.....V...AL...R...V	74%
4. Hox B2 (<i>Mus musculus</i>) [A35511]		F.K.....V...AL...R...V	74%
5. Hox 2 (<i>Saccoglossus kowalevskii</i>) [C44636]		K.....SM...S...R...V	78%
anthox4-Ms		LYSRYLTRTRLEAKSLGLSEKHLKI	
1. Hox C4 (<i>Rattus norvegicus</i>) [P18865]		H.N.....R...I.I.H.C...R.QI..	63%
2. Hox 8 (<i>Branchiostoma floridae</i>) [I44629]		HFN.....R...I.I.H.A...R.QI..	59%
3. Hox 5 (<i>Saccoglossus kowalevskii</i>) [F44636]		HFN.....R...I.I.H.A...R.QI..	59%
4. Hox 4 (<i>Petromyzon marinus</i>) [H48200]		HFN.....R...L.I.H.A...R.QV..	63%
5. HB3 (<i>Tripneustes gratilla</i>) [P10178]		HF.....V...R.F.I.Q...R.QI..	63%
anthox5-Ms		TKNNYLTRLRYYEIAMGLDLAERQVKV	
1. Cnox5 (<i>Hydra magnipapillata</i>) [S39068]		VR.....VS.S.S.....	78%
2. mox-2 (<i>Mus musculus</i>) [S29902]		AHH.....VN...T.....	78%
3. homeotic protein (<i>Dugesia tigrina</i>) [S32729]		AVH.....L.VA.N.N...I.....	67%
4. btn gene product (<i>Drosophila</i>) [S75228]		CYS.....VA.E.T.....	74%
5. mox-1 (<i>Mus musculus</i>) [P32442]		AHH.....VN...S.....	78%
anth-eye-Ms		LRENVSRTTRCELANSLSLSETTIKI	
1. eveC (<i>Acropora formosa</i>) [S36770]		S.....SM.N.....	85%
2. Xhox-3 (<i>Xenopus laevis</i>) [A60092]		Y.....P.....AA.N.P...V	74%
3. eve (<i>Drosophila melanogaster</i>) [X05138]		YK.....P.....AQ.N.P.S...V	67%
4. EVX2 (<i>Homo sapiens</i>) [M59983]		Y.....P.....AA.N.P...V	74%
5. EVX1 (<i>Homo sapiens</i>) [S22586]		Y.....P.....AA.N.P...V	74%
anth-eye-Nv		LMRENVSRTTRCELANALNLSSETTIKI	
1. eveC (<i>Acropora formosa</i>) [S36770]		L.....S.....SM.....	85%
2. Xhox-3 (<i>Xenopus laevis</i>) [A60092]		Y.....P.....AA.P...V	78%
3. EVX2 (<i>Homo sapiens</i>) [M59983]		Y.....P.....AA.P...V	78%
4. eve (<i>Drosophila melanogaster</i>) [X05138]		YK.....P.....AQ...P.S...V	70%
5. EVX1 (<i>Homo sapiens</i>) [S22586]		Y.....P.....AA...P...V	78%
anth-hbxA-Ms		GEKYLTEAKRAELSKDLGMTQVKT	
1. Eghbx2 (<i>Echinococcus granulosus</i>) [JC1387]		DRQ...SS.E...M.R...LS...I	59%
2. homeo A-1 (<i>Echinostoma trivolvis</i>) [L19170]		HFNR...R.R.I.I.AN...L...I	56%
3. engrailed (<i>Drosophila melanogaster</i>) [K03059]		N.NR...RR.QQ...SE.LN.A.I.I	52%
4. homeobox (<i>Helicidaris erythrogramma</i>) [U20895]		KAD...SVS...L...MS.NL...I	56%
5. E30 (<i>Apis mellifera</i>) [P09076]		A.NR...RR.QQ...R...L.A.I.I	56%
anth-hbxA-Nv		KEEKYLTEKRAELSKDLGMTQVKT	
1. Eghbx2 (<i>Echinococcus granulosus</i>) [JC1387]		DRQ...SSAE...M.R...LS...I	56%
2. homeobox (<i>Helicidaris erythrogramma</i>) [U20895]		.AD...SV...L...MS.NL...I	63%
3. E30 (<i>Apis mellifera</i>) [P09076]		A.NR...RR.QQ...R...L.A.I.I	56%
4. engrailed (<i>Drosophila melanogaster</i>) [K03059]		N.NR...RR.QQ...SE.LN.A.I.I	52%
5. homeo A-1 (<i>Echinostoma trivolvis</i>) [L19170]		HFNR...R.R.I.I.AN...L...I	52%
anth-hbxB-Ms		ERQYVMGAERHYLAASLNLTEQVVKV	
1. EMX2 (<i>Mus musculus</i>) [Q04744]		.KNH.V...KQ...H.S.....	70%
2. EMX1 (<i>Mus musculus</i>) [Q04742]		.KNH.V...KQ...G.S.S.....	70%
3. Cnot 1 (<i>Gallus gallus</i>) [X82575]		LK.....T.VD...T.R.....	67%
4. Xnot 1 (<i>Xenopus laevis</i>) [A46305]		LK.....T.VD...ST.....	74%
5. EMX2 (<i>Homo sapiens</i>) [Q04743]		.KNH.V...KQ...H.S.....	70%
anth-hbxC-Nv		EAKKYLATERSDMASLLNVTEQVVKI	
1. Hox A8 (<i>Branchiostoma floridae</i>) [L14867]		.V...S.S...A.L.A...D...RK	67%
2. ceb-9 (<i>Caenorhabditis elegans</i>) [S13129]		SSSD...EL.KR.D.....	67%
3. msx-2 (<i>Mus musculus</i>) [PS0410]		RQ.Q...SIA...AEFS.S.L.....	56%
4. H17 (<i>Apis mellifera</i>) [P15857]		RE.Q...IA...AEFS.S.HL.....	56%
5. msx-2 (<i>Gallus gallus</i>) [H45187]		RQ.Q...SIA...AEFS.S.L.....	56%

Felsenstein, 1989; Fig. 2), and a parsimony tree was constructed using the program *PAUP* (Phylogenetic Analysis Using Parsimony; version 3.1; Swofford, 1991; Fig. 3).

On the gene phylogenies (Figs. 2 and 3), Class I, Class II, and Class IV, as defined by Naito *et al.* (1993; Table I) are monophyletic. Among the anemone homeodomains, *anthox1* of *Metridium* seems to be orthologous to *anthox1* of *Nematostella*. *Anth-eve* of *Metridium* and *Nematostella* also appear orthologous, as do *anth-hbxA* of *Metridium* and *Nematostella*. The phylogenies also permit a tentative assignment of some of the anemone homeodomains to specific cnidarian classes. *Anth-eve* appears orthologous to *eveCoral* (Class VII). *Anthox1* and *anthox1a* cluster with members of cnidarian Class I that are believed to be related to the *Antennapedia*-like, or Hox class, homeoboxes (Naito *et al.*, 1993). *Anthox5* appears orthologous to *cnox5* (Class V). Other associations appear more tenuous.

Identity of anemone homeoboxes assessed by searching the Genbank database

The sequences of anemone homeodomains inferred from the gene fragments recovered in this study were compared to sequences in GenBank with the BLASTx network search algorithm (Basic Local Alignment Search Tool; Altschul *et al.*, 1990). The top five matches recovered in the BLAST searches are aligned to their anemone homeodomain counterparts in Figure 4. For each of the unique anemone homeodomain fragments, the search was limited to the 50 most similar genes in the GenBank database. In every case, all 50 of the genes identified were homeobox genes. At the amino acid level, anemone homeodomains are 59%–85% identical to the most similar homeobox genes in the GenBank database. The BLAST searches reveal that five of the anemone sequences are most similar to homeodomains of the Hox class: these are *anthox1*, *anthox1a*, *anthox2*, *anthox4*, and *anthox5*. The *anth-eve* homeodomains are most similar to *even-skipped* homologs from other species, particularly the *even-skipped* homolog of the staghorn coral (Miles and Miller, 1992). The *anth-hbxA* homeodomains from both *Metridium* and *Nematostella* appear most similar to the flatworm homeodomain *EgHbx2* (Oliver *et al.*, 1992). *EgHbx2* has been referred to as an NK type homeobox, but this assignment is not supported by a recent homeobox gene tree (Bürglin, 1994). The *anth-hbxA* homeodomains also resemble the *engrailed*

homeodomain of *Drosophila*. *Anth-hbxB* of *Metridium* is most similar to *empty-spiracles* homologs from the mouse (EMX1 and EMX2). *Anth-hbxC* from *Nematostella* exhibits similarity to a diverse group of genes, including Hox A8 from *Branchiostoma* and *msx2* from mouse, two members of the *msh*-class of homeodomains.

Phylogenetic relationships of homeoboxes from sea anemones and higher metazoans

Phylogenetic analyses of cnidarians and bilaterians were used to further assess the identity of the anemone homeoboxes (Saitou and Nei, 1987). In addition to the 12 anemone homeodomains and 13 sequences from other cnidarians, our analysis included 20 sequences from bilaterian animals, each representing a different class of homeodomains (Bürglin, 1994). A neighbor-joining tree (Fig. 5) supports the finding that some of the anemone homeoboxes belong to previously proposed cnidarian classes (Naito *et al.*, 1993; see previous section *Comparison of homeoboxes from anemones and other cnidarians*). *Anthox1* allies with members of Class I (*cnox1.Hm* and *S.Aox3*), *anthox2* with members of Class II (*cnox2.Cv* and *S.Aox2*), *anthox4* with members of Class IV (*cnox4.Hm* and *cnox1.Cv*), *anthox5* with Class V (*cnox5.Hm*), and *anth-eve* with Class VII (*eveC*). All of these findings, with the exception of the precise relationships of *anthox4*, are supported by a parsimony analysis (Fig. 6).

The phylogenetic analyses suggest that certain anemone homeoboxes represent classes previously unidentified in the Cnidaria (Figs. 5 and 6). *Anthox1a* from *Nematostella* appears as the sister group to the Class I genes and may represent a new class of cnidarian homeoboxes (Class Ia). *Anth-hbxA*, found in both *Metridium* and *Nematostella*, appears most closely related to the homeobox from the human gene TCL-3, a non-cluster member of the Hexapeptide superclass (Bürglin, 1994). As suggested by the BLASTx search results (Fig. 3), *Anth-hbxB* from *Metridium* appears most closely related to the *Drosophila empty-spiracles* homeobox, a gene known to be involved in head development in both *Drosophila* and vertebrates (Walldorf and Gehring, 1992; Simeone *et al.*, 1992). Finally, *Anth-hbxC* appears most closely related to the *C. elegans* homeobox *ceh-9*. The PCR surveys of sea anemones failed to recover representatives of cnidarian classes 3, 4, 6, or 8. The BLAST searches and phy-

Figure 4. Alignments of anemone homeodomain fragments to similar homeodomains identified by searching the GenBank molecular database with the BLASTx algorithm (Basic Local Alignment Search Tool; Altschul *et al.*, 1990). The predicted amino acid sequences of anemone homeodomains (amino acids 21–47) are aligned to the five best matches identified by the database search in order of decreasing similarity to the query sequence. Periods indicate identity with the anemone homeodomain. Accession numbers of GenBank sequences are presented in brackets.

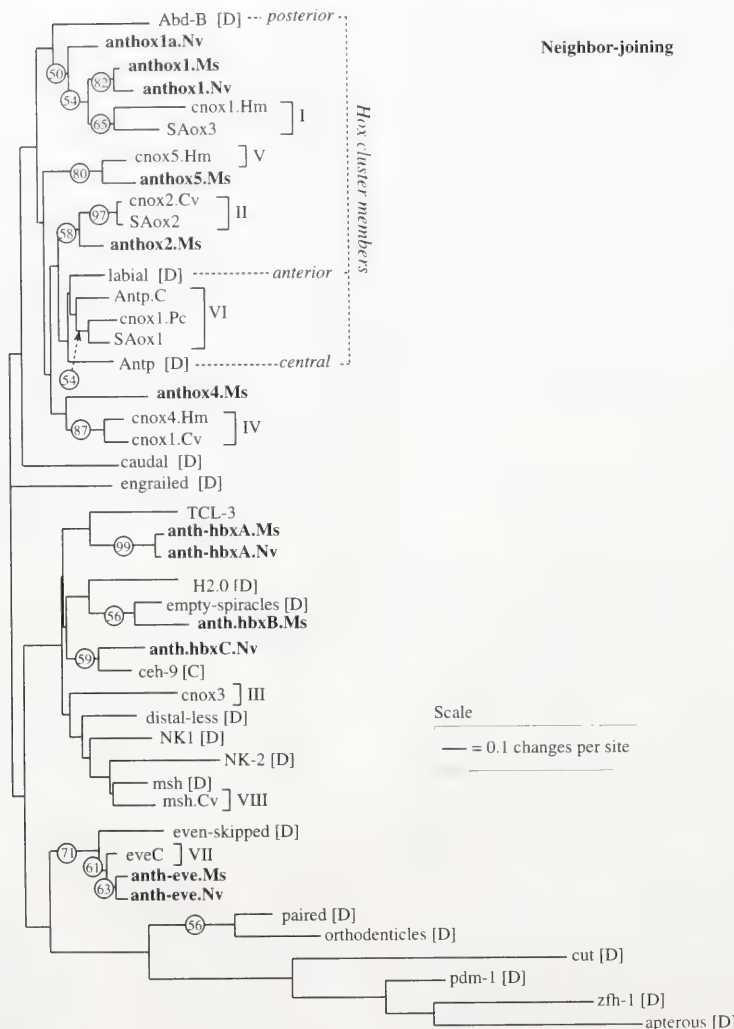


Figure 5. Phylogenetic relationships of predicted homeodomain sequences from cnidarians and bilateria: s were inferred using the neighbor-joining algorithm (Saitou and Nei, 1987). The tree was generated as described in Figure 2. Cnidarian homeodomain sequences are labeled as in Figures 2 and 3, except that brackets are used to identify representative sequences from each cnidarian class (Naito *et al.*, 1993; roman numerals). Non-cnidarian homeodomain sequences were chosen to represent each of the 20 proposed classes of homeobox genes that make up an existing classification scheme (Bürglin, 1994). Non-cnidarian sequences are from *Drosophila* [D], *C. elegans* [C], and *Homo sapiens* [H]. Branch lengths in the horizontal direction are proportional to the number of expected amino acid substitutions that have occurred per site (scale at lower right). Branch lengths in the vertical direction are not significant. Circled numbers along branches indicate the percentage of trials in which a given partition between genes was found in 2000 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >40% of bootstrap replicates are indicated. Dashed lines indicate homeoboxes of the Hox class from *Drosophila*. These genes have differing anterior borders of expression along the anterior-posterior axis of the developing embryo. *Labial* is expressed more anteriorly than *Antennapedia* (*Antp*), which in turn is expressed more anteriorly than *AbdominalB* (*AbdB*). For this reason, Hox genes are sometimes classified according to their expression pattern in the embryo as anterior (e.g., *labial*), central (e.g., *Antennapedia*), and posterior (e.g., *AbdominalB*).

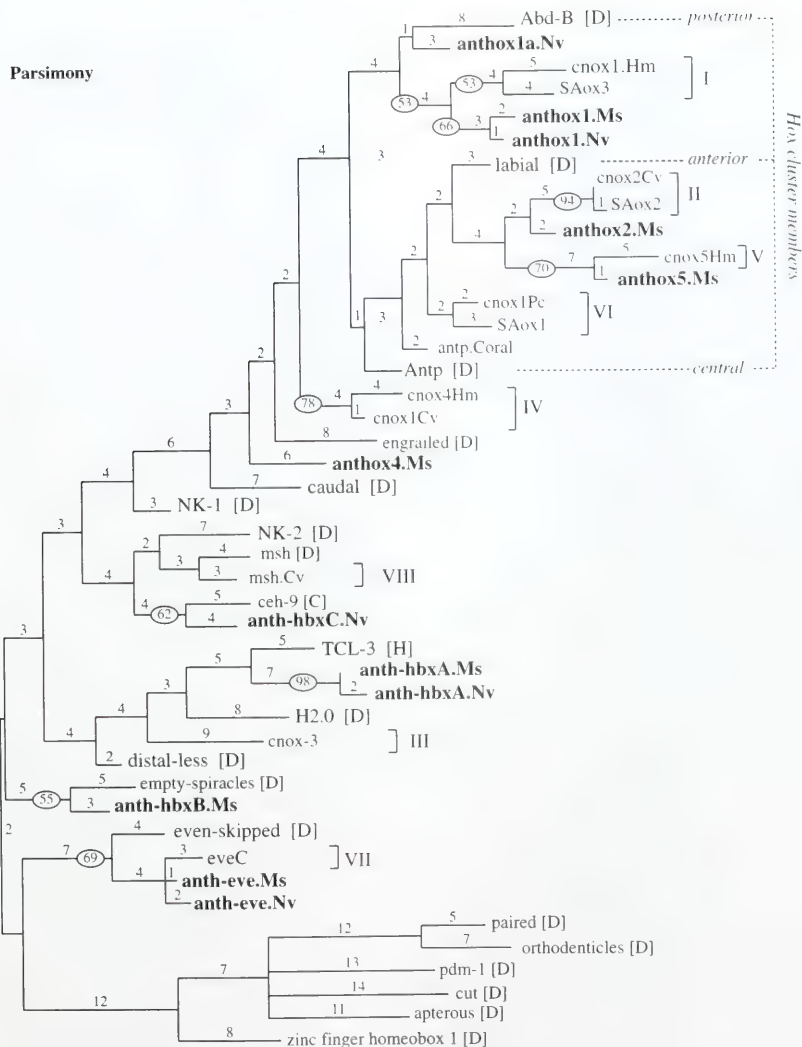


Figure 6. Phylogenetic relationships of predicted homeodomain sequences from cnidarians and bilaterians were inferred using the maximum parsimony criterion. The tree was generated as in Figure 3. Sequences used are identical to those in Figure 5. Anemone homeodomains are indicated in boldface type. The tree depicted is a 50% majority-rule consensus of 123 equally parsimonious trees identified by tree-bisection and reconnection (TBR) branch swapping on 20 random starting trees. Tree length = 354 steps. Consistency index (CI) = 0.506. Retention index (RI) = 0.557. Branch lengths in the horizontal direction are proportional to the number of amino acid substitutions that have occurred along each branch. The number of amino acid substitutions is specified above each branch. Branch lengths in the vertical direction are not significant. Circled numbers indicate the percentage of trials in which a given partition between genes was found in 200 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >50% of bootstrap replicates are indicated.

Table II
Proposed classification of cnidarian homeoboxes based on BLAST searches and phylogenetic analysis

Species	Homeobox Class										
	I Hox	Ia Hox	II Hox	III dll/S59	IV Hox	V Hox	VI Hox	VII even-skipped	VIII msh	IX TCL-3	X empty-spiracles
Cnidarian											
Bilaterian†											XI eeh-9
Class Hydrozoa											
<i>Chlorohydra viridissima</i>			<i>cnmx2</i>	<i>cnmx3</i>	<i>cnmx1</i>				<i>msh C^v</i>		
<i>Eleutheria dichotoma</i>	<i>cnmx1</i>		<i>cnmx2*</i>								
<i>Hydra magnipapillata</i>	<i>cnmx1</i>		<i>cnmx2*</i>		<i>cnmx4</i>	<i>cnmx5</i>					
<i>Hydra vulgaris</i>			<i>cnmx2*</i>								
<i>Hydractinia</i>			<i>cnmx2*</i>								
<i>Symbiolonicarpus</i>			<i>cnmx2*</i>				<i>cnmx1</i>				
<i>Podocoryne carnea</i>			<i>S4ox2</i>				<i>S4ox1</i>				
<i>Sarsia</i>	<i>S4ox3</i>										
Class Anthozoa											
<i>Metridium senile</i>	<i>anthox1</i>		<i>anthox2</i>		<i>anthox4**</i>	<i>anthox5</i>		<i>anth-ene</i>		<i>anth-lhvx4</i>	<i>anth-lhvxB</i>
<i>Nematostella vectensis</i>	<i>anthox1</i>	<i>anthox1a</i>					<i>anipC</i>	<i>anth-ene</i>		<i>anth-lhvx4</i>	<i>anth-lhvxC</i>
<i>Acropora formosa</i>								<i>eevC</i>			

This classification scheme for cnidarian homeoboxes builds on that suggested by Naito et al. (1993) and is consistent with the results of BLAST searches (Fig. 4) and phylogenetic analyses (Figs. 5 and 6).

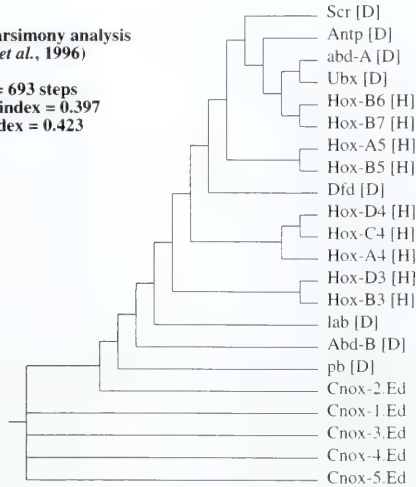
† The bilaterian classification scheme is taken from Burgin (1994). Homology between cnidarian and bilaterian classes is based on the phylogenetic analyses (Figs. 5 and 6). New cnidarian classes suggested by the sea anemone data are Classes Ia, IX, X, and XI.

* *Cnox2* from *Eleutheria dichotoma*, *Hydra magnipapillata*, *Hydra vulgaris*, and *Hydractinia symbiolonicarpus* was not included in the phylogenetic analyses because the homeodomains have predicted amino acid sequence identical to that of *cnmx2* from *Chlorohydra viridissima*.

** The neighbor-joining tree (Fig. 5) supports inclusion of *anthox4* in class IV, whereas the parsimony tree (Fig. 6) suggests that *anthox4* represents a novel class.

A. Nucleotide parsimony analysis
(from Kuhn *et al.*, 1996)

Tree length = 693 steps
Consistency index = 0.397
Retention index = 0.423



B. Amino acid parsimony analysis

Tree length = 184 steps
Consistency index = 0.723
Retention index = 0.638

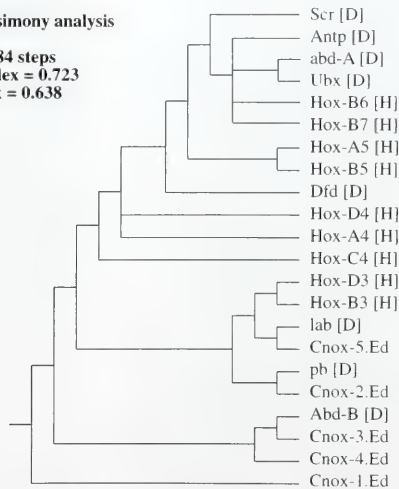


Figure 7. Comparison of parsimony analyses based on nucleotide sequences and amino acid sequences. (A) Kuhn *et al.* (1996) inferred the phylogenetic relationships of cnidarian hox gene fragments (*cnox1-cnox5* of *Eleutheria dichotoma*) to HOM/Hox homeoboxes from humans and *Drosophila* using parsimony. The tree depicted is a 50% majority-rule consensus of eight equally parsimonious trees found in a heuristic search based on unweighted nucleotide sites (180 base pairs); branch swapping by subtree-pruning-regrafting was performed on 100 random starting trees. (B) A reanalysis based on a parsimony analysis of unweighted amino acid sites (60 sites) using identical methods to search for the most parsimonious tree.

logenetic analyses suggest to us an expanded working classification of homeobox genes in the Cnidaria (Naito *et al.*, 1993) to encompass the new data from sea anemones (Table II).

The Hox class in basal metazoans

In distantly related taxa, such as vertebrates, insects, and nematodes, the genes of the Hox class are located in

evolutionarily conserved genomic clusters. Furthermore, individual Hox genes are expressed along the anterior-posterior axis of the developing embryo in the same relative order as their position in the Hox cluster: genes located towards the 3' end of the cluster are expressed more anteriorly than genes located towards the 5' end of the cluster. This evidence suggests that a linked cluster of Hox genes is a shared, derived metazoan character that is involved in patterning the anterior-posterior axis (Slack *et al.*, 1993; Miller and Miles, 1993). However, very little is known about the composition of the Hox cluster in basal metazoans such as the Cnidaria, or even whether such a cluster exists.

As our knowledge of Hox genes in basal metazoans increases, we should be able to establish the antiquity of the Hox cluster and of individual Hox genes, and to make inferences about the primitive function of Hox genes. Among the relevant questions are (1) How many Hox genes are found in the Cnidaria? and (2) What are the relationships of cnidarian Hox genes to Hox genes from bilaterian animals? Phylogenetic analyses of vertebrate and insect Hox genes suggest a very early trichotomy in the evolution of the Hox class—giving rise to an *Antennapedia/deformed* precursor, a *labial/proboscidia* precursor, and an *AbdominalB* precursor (Schubert *et al.*, 1993). Previous studies revealed similarities between individual cnidarian homeoboxes and homeoboxes from the *Drosophila* genes *labial*, *proboscipedia*, *Deformed*, and *Antennapedia* (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Schummer *et al.*, 1992; Naito *et al.*, 1993). These homeobox genes have been referred to as anterior and central members of the *Drosophila* HOM/Hox cluster because their anterior borders of expression lie in the anterior half of the *Drosophila* embryo (Bartels *et al.*, 1993). A homolog of the posterior Hox genes (*AbdominalB*-like) has not been recognized in the Cnidaria.

With few exceptions (*e.g.*, Naito *et al.*, 1993), the identities of cnidarian Hox genes have been considered in isolation: Hox class genes cloned from a single species are compared to Hox sequences from *Drosophila* and vertebrates without reference to Hox genes from other cnidarians or to non-Hox homeoboxes; these comparisons have been made using pairwise alignments (*e.g.*, Schummer *et al.*, 1992; Miles and Miller, 1992; Miller and Miles, 1993) or, more rarely, phylogenetic techniques (Aerne *et al.*, 1995; Kuhn *et al.*, 1996). In this study we have attempted a more systematic approach: we used phylogenetic techniques to simultaneously assess the relationships between all the reported cnidarian homeoboxes and a broad representation of homeoboxes from bilaterian animals. As in several previous studies, our phylogenetic analyses suggest that certain cnidarian Hox genes are closely related to anterior and central members of the Hox cluster in higher metazoans (Murtha *et al.*,

1991; Schierwater *et al.*, 1991; Schummer *et al.*, 1992; Naito *et al.*, 1993). The members of cnidarian classes II, IV, V, and VI appear to be more closely related to the *labial* and *Antennapedia* homeoboxes of *Drosophila* than to the *AbdominalB* homeobox or any non-Hox homeobox. Our analyses indicate that cnidarians may possess homologs to posterior members of the complex as well as to the more anterior genes. The phylogenetic analyses (Figs. 5 and 6) suggest that members of the cnidarian homeobox classes I and Ia are most closely related to the *Drosophila Abdominal-B* homeobox, the most posterior member of the *Drosophila* Hox cluster. Therefore, distinct anterior/central (*Antennapedia/labial*-like) and posterior (*AbdominalB*-like) Hox gene lineages may have predated the split between the Cnidaria and the Bilateria (Schubert *et al.*, 1993). Additional data on cnidarian Hox genes, such as their genomic organization and axial expression, may serve to corroborate or contradict this hypothesis.

A recent paper by Kuhn *et al.* (1996) suggested a very different scenario for the origin of the HOM/Hox genes. Hox genes from triploblasts (*Drosophila* and humans) were compared with Hox genes from a cnidarian (the hydrozoan *Eleutheria dichotoma*) by using parsimony and distance methods. In the phylogenetic analyses, the resulting topologies can be rooted such that genes from triploblasts form a monophyletic group to the exclusion of all cnidarian Hox-type genes, suggesting that the common ancestor of triploblasts and cnidarians possessed only a single Hox-type gene. In other words, Kuhn *et al.* (1996) find no evidence of a Hox cluster in the common ancestor of diploblasts and triploblasts, whereas the analyses presented here are consistent with the existence of an ancestral cluster.

The difference in the two phylogenetic conclusions derives from the choice of phylogenetic characters—the analyses reported here were based on the inferred amino acid residues within the homeodomain; those of Kuhn *et al.* (1996) were based on the entire nucleotide sequence of the homeobox, including rapidly evolving, silent nucleotide sites within codons. But the relevant evolutionary time scale is very long: both cnidarian and bilaterian fossils are known from Cambrian sediments laid down more than 500 million years ago (*e.g.*, Sepkoski, 1981), so more than 1 billion years of independent evolution separate the last common ancestor of modern-day triploblasts and cnidarians. Therefore, rapidly evolving sites are likely to be saturated with phylogenetic noise. Furthermore, if there are differences in codon bias between taxa, the inclusion of silent sites in the analysis may falsely indicate phylogenetic affinity between non-homologous gene sequences derived from closely related taxa. When the phylogenetic analysis of Kuhn *et al.*, (1996) is repeated with the inferred amino acid substitu-

tions as phylogenetic characters, the result is consistent with the existence of an ancestral cluster (Fig. 7).

Confidence in inferring orthology of homeodomain sequences

In trying to infer the evolutionary relationships of homeobox genes, we are confronted by historical limitations. Our inferences are based on a small region of sequence that is highly conserved across very distantly related phyla. Sequence conservation outside the homeodomain is generally poor, so sequence alignment is difficult. The phylogenetic utility of sequences outside the homeodomain may therefore be limited. Considering this historical limitation, our inferences about the identity of several of the cnidarian homeodomains appear surprisingly robust. Several partitions on the neighbor-joining tree that suggest homology between cnidarian and bilaterian homeodomains are supported by more than 50% of bootstrap trials (for example, cnidarian *even-skipped* homologs + *Drosophila eve*: bootstrap proportion $\geq 75\%$). Results from computer simulations and experimental bacteriophage phylogenies suggest that bootstrap proportions over 50% can indicate a much higher than 50% probability that the corresponding branch is correct (Hillis and Bull, 1993; Felsenstein and Kishino, 1993). The results presented here agree with an emerging pattern: newly discovered homeoboxes can often be assigned to previously described classes with reasonable confidence, though the interrelationships between different classes of homeobox genes are difficult to reconstruct (e.g., Finnerty *et al.*, 1996). This pattern suggests that many of the homeobox classes are extremely ancient, and that the sequences of these genes have been very strongly conserved.

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An *in vivo* Comparative Study of Intersegmental Flexibility in the Ophiuroid Arm

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Abstract. We present the first *in vivo* measurements of intersegmental rotation in the ophiuroid arm, comparing lateral bending performance in seven epifaunal species from Discovery Bay, Jamaica. The species studied include suspension-feeders, deposit-feeders, and scavengers, and also represent two major types of vertebral ossicle morphology. Animals were photographed with strobe illumination, and the angular deflections between arm segments were recorded. Despite considerable variation in vertebral morphology, ecology, and behavior, Discovery Bay ophiuroids show similar, overlapping distributions of maximal intersegmental rotations. Although interspecific differences in mean lateral flexibility can be statistically significant, absolute differences among species are small and of unknown functional significance. These quantitative data challenge long-standing assumptions about how the ophiuroid vertebral skeleton affects intersegmental flexibility, and how intersegmental flexibility *per se* affects an ophiuroid's ecological style or success.

Introduction

The echinoderm skeleton, like all skeletal systems, constrains and directs the actions of the animal. The skeletal ossicles can be connected rigidly, as in the echinoid test, or flexibly, as in the mobile appendages of crinoids, asteroids, and ophiuroids. Each type of connec-

tion imposes certain biomechanical limits on organismal form and function. Only a few of these systems, however, have been studied in detail (crinoids: Meyer, 1973; Macurda and Meyer, 1976; Donovan, 1988; Riddle *et al.*, 1988; Holland *et al.*, 1991; Baumiller *et al.*, 1991; Baumiller and LaBarbera, 1993; echinoids: Telford, 1985; Eilers and Telford, 1991; Carnevali *et al.*, 1991, 1993; asteroids: Eylers, 1976; O'Neill, 1989).

The behavior and ecology of ophiuroids are thought to be influenced by the mobility of their heavily calcified, multisegmented arms (Fig. 1). Externally, the ophiuroid arm is covered with a regular arrangement of overlapping plates or scales. Internally, the arm contains a series of disk- or rod-shaped vertebral ossicles that articulate with one another proximally and distally, forming a central "spine" that allows the arm to bend under muscular control (for review, see Byrne, 1994). Coordinated motion at numerous intervertebral joints gives the ophiuroid arm an exceptional range of flexibility, as seen in many locomotory and food-gathering behaviors (Fell, 1966; Magnus, 1967; Pentreath, 1970; Warner, 1982; for review, see Lawrence, 1987). Although the shape of the vertebral ossicle is widely presumed (see below) to affect intervertebral rotation, intersegmental flexibility, and thus the potential behavior and ecology of the animal, the range of vertebral morphologies is little studied and their effects remain largely untested.

Ophiuroids are the most ecologically diverse echinoderm class, encompassing predatory, scavenging, suspension-feeding and deposit-feeding lifestyles (for reviews, see Warner, 1982; Lawrence, 1987). This "high ecological adaptability" (Litvinova, 1989a) has been attributed to the evolutionary innovation of the vertebral ossicle system, which presumably allowed increased arm

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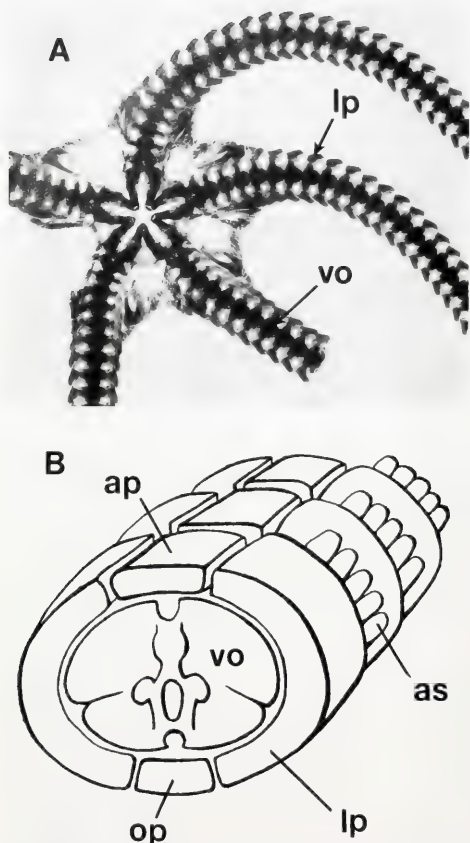


Figure 1. Anatomical overview of the ophiroid arm skeleton. (A) X-ray view showing segmentation of the arms and the internal series of vertebral ossicles (*Ophioderma cinereum*). (B) Diagrammatic cross-section of the arm showing the relationships of internal and external skeletal plates. ap = aboral plate, as = arm spine, lp = lateral plate, op = oral plate, vo = vertebral ossicle.

mobility (Spencer and Wright, 1966) and the subsequent "radiation" of vertebral ossicle morphologies within the established arm skeleton. Thus vertebral variations among both living and fossil ophiroid taxa are often described as mechanical adaptations for different lifestyles or behaviors (Berry, 1934; Litvinova, 1989a,b).

Such variations have long been noted (Lyman, 1882; Bell, 1892; Matsumoto, 1915, 1917), and recent research has explored the taxonomic distribution and functional implications of different vertebral ossicle forms (Emson

and Wilkie, 1982; Robbins, 1986; Bray, 1988; Byrne and Hendler, 1988; Litvinova, 1989a,b; Hendler and Miller, 1991; LeClair, 1994, 1995, 1996). These descriptive studies generally focus on the shape of the intervertebral articulation, which is often assumed to influence rotation between arm segments. Each vertebral ossicle has two articulation surfaces, one proximal and one distal. In basket stars (order Euryalae), these surfaces have an hourglass-shaped, streptospondylous articulation, which allows the arm to coil helically for maintaining posture and obtaining food (Macurda, 1976; Hendler and Miller, 1984; Dearborn *et al.*, 1986; Emson *et al.*, 1991). Other taxa (order Ophiurae) show a more complex zygospondylous articulation, in which each surface bears a bilaterally symmetrical set of projections and depressions (Fig. 2). The zygospondylous joint is assumed to be more limited in motion, rotating primarily in a lateral plane (Hyman, 1955; Byrne, 1994). The ophiurans are taxonomically and ecologically more diverse than the euryalans, a fact attributed to greater diversity in the configuration of the zygospondylous joint (*e.g.*, Hendler and Miller, 1991).

Interspecific variations in ophiuran vertebral ossicle morphology have been linked to unusual forms of locomotion (Hendler and Miller, 1991) and to specific feeding strategies (Robbins, 1986). Emson and Wilkie (1982) interpreted certain vertebral features in the Amphiprionidae, Ophiactidae, and Ophiotrichidae as modifications for increased flexibility and postural control, and concluded that "arm structure may be correlated with habit, and also with habitat" (p. 17). Bray (1988) described differences between the vertebral ossicles of *Ophiocoma echinata* and *Ophiotrichus suensonii*, and how these variations might influence arm movement and ecology. Hendler and Miller (1991) suggested that reduced articular surfaces in certain *Ophiotrichus* might allow a greater range of motion between arm segments, facilitating open-water swimming behavior. The most detailed discussions of the functional morphology of the vertebral ossicle system are by Litvinova (1989a,b; 1994), who inferred mechanical consequences for a variety of vertebral ossicle types.

The ophiroid literature thus contains many working hypotheses about the biomechanics of the intervertebral articulation, its influence on intersegmental flexibility, and how this flexibility may correlate with ecologically interesting behavior. These hypotheses generally assume (1) that the degree of bending between arm segments depends on the morphology of the intervertebral articulation and (2) that the behavior or ecology of the animal depends on the degree of bending between arm segments. Although structural differences are thus used to explain interspecific differences in intersegmental flexi-

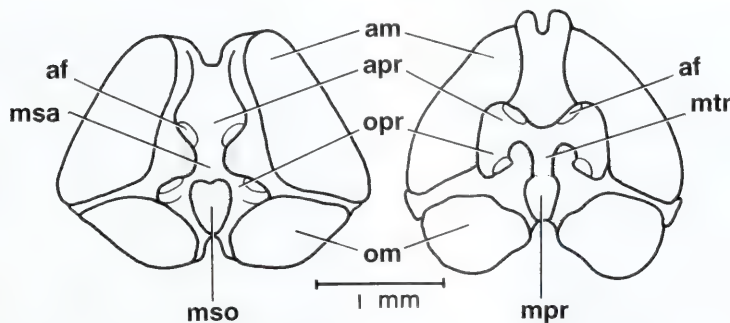


Figure 2. Proximal (left) and distal (right) articulating surfaces of a zygospondylous vertebral ossicle. af = articulating facet, am = aboral muscle area, apr = aboral process, mpr = median process, msa = median saddle, msso = median socket, om = oral muscle area, opr = oral process. Diagram and descriptive terminology slightly modified from Bray (1985).

bility and ecology, no study has quantified the relative flexibility of different ophiroid arm joints.

Here we report the first comparative assessment of ophiroid intersegmental flexibility *in vivo*. The first goal of our investigation was to measure intersegmental rotations in live, unrestrained animals representing seven ophiurid families and two major vertebral ossicle morphologies. The second goal was to explore how any differences in observed flexibility might correlate with vertebral ossicle shape, taxonomic group, or dominant feeding mode of the animal. If vertebral ossicle morphology is the primary factor dictating intersegmental flexibility, species with very different vertebral morphologies are predicted to show measurable differences in intersegmental rotations. Conversely, without evidence for interspecific variation in arm performance at the level of the segment, it is unnecessary to invoke differences in vertebral morphology as a causal or adaptive explanation.

Materials and Methods

Experimental animals

Epifaunal ophiroids were collected from backreef areas at Discovery Bay Marine Laboratories, Discovery Bay, Jamaica (March–April 1995; Fig. 3A). This area supports at least 15 species of ophiroids, representing 7 families in the order Ophiurae (Sides, 1981). Animals were captured by hand during snorkel and scuba surveys at four sites previously described by Sides (1981) and at a fifth location immediately north of the laboratory's rock jetty (Fig. 3B). Large individuals of *Ophioderma cinereum* were abundant at the latter site, and *Ophiothrix oer-*

stedii was also common. Several individuals of *Ophiothrix suensonii* were collected by scuba divers at Red Buoy Reef in the eastern half of the bay (depth = 18–20 m). Unlike the backreef taxa, which tend to be cryptic during the day, this species was usually conspicuous: individuals could be seen resting, with several arms exposed, in the mouths of large cup sponges.

Live ophiroids were transferred immediately to running seawater tables for the duration of the field session (2–3 weeks). All animals appeared healthy and active and no arm autotomy was observed. To prevent the animals from becoming acclimated to light, the seawater tables were kept dark with black plastic curtains. No food was added to the tanks, but small particles and sediment were introduced by the constant flow of seawater. All ophiroids were allowed to adjust for at least 24 h before they were used in any behavioral experiments. Table I lists the species collected, the number of individuals used, and the size of each individual as measured by disk diameter (in millimeters).

The taxa studied also represent a diversity of feeding strategies, including scavenging (*Ophioderma appressum*, *O. cinereum*), deposit-feeding (*Ophiocoma pumila*), obligate suspension-feeding (*Ophiothrix oerstedii*, *O. suensonii*), and facultative deposit-/suspension-feeding (*Ophiocoma echinata*, *O. pumila*). These behavioral categorizations are drawn from previous studies of the same species at the same locality (Sides, 1981, 1984; Sides and Woodley, 1985). Table II lists the vertebral ossicle morphologies and predominant feeding strategies of the species sampled. Two vertebral types, keeled and non-keeled, are represented. A detailed description of these types and an analysis of variation within types is

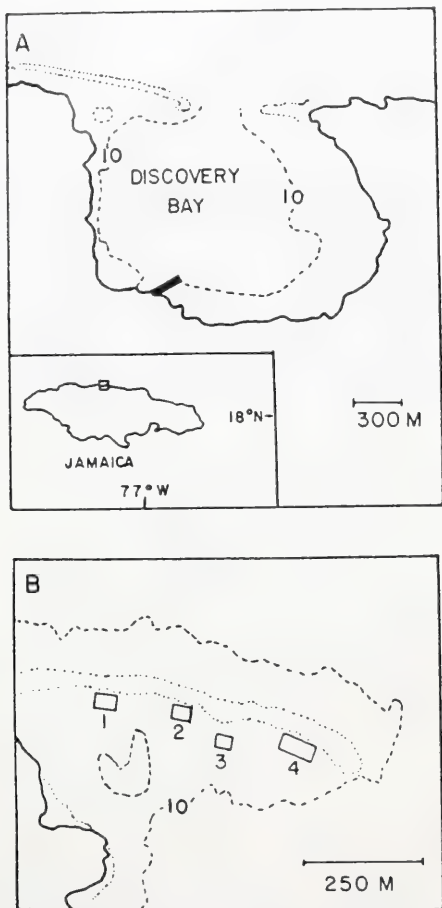


Figure 3. Map of Discovery Bay, Jamaica (from Sides, 1981). (A) Overview of the bay. Dotted line = 10-meter depth contour. (B) Detail of the western half of the bay showing backreef collecting sites (1–4).

given elsewhere (LeClair, 1994, 1995, 1996; see also Robbins, 1986; Smith *et al.*, 1995). In contrast to non-keeled vertebral ossicles (Fig. 4A), keeled vertebral ossicles have reduced articulating projections, a large notch on the proximal aboral surface, and an aboral, distal-pointing projection, or “keel” (Fig. 4B). Each distal keel fits into the proximal notch of the adjacent vertebral ossicle, creating an overlapping, or “imbricated,” skeletal series that non-keeled morphs lack (Fig. 4C). Keeled taxa

also feature “accessory aboral muscles” between the proximal depression and distal keel that are absent in non-keeled taxa (Fig. 4B; Robbins, 1986). This dichotomy in skeletal structure led us to predict that species with different vertebral ossicle morphologies would show measurable differences in intersegmental flexibility—specifically, that the reduced articular surfaces and additional muscles present in keeled species would allow greater lateral bending, as proposed by Hendler and Miller (1991).

Photographic apparatus

Intersegmental flexibility was recorded by photographing unrestrained ophiuroids moving in a shallow glass aquarium (30 × 23 × 8 cm). A front-surface mirror was mounted underneath the aquarium, angled 45° to the tank bottom. A Nikon 6006 camera equipped with a macro lens was aimed at this mirror to provide a view perpendicular to the tank floor and the oral surface of the animal. Two Vivitar strobe lights were angled upwards beneath the tank to briefly (1/10,000 s) illuminate the animal from below during each exposure (1/125 s). All exposures were recorded on Kodak Tech Pan film (ASA 25). Images obtained by this method show excellent detail of the oral arm surface and good contrast at the boundaries between external plates (Fig. 5).

Arm-flexing behaviors

All experiments were conducted in a darkened laboratory after dusk (1830–0200 h) when these species are most active (Sides, 1981). Ophiuroids generally show negative phototaxis, rapidly crawling away from any light source. This tendency was exploited to elicit two types of arm-flexing behaviors in the laboratory:

Coiling: One ophiuroid was placed in the center of the tank next to a small, slightly concave rock or coral chip. Most animals, with some prodding, quickly occupied this artificial crevice. Once inside, the ophiuroid was illuminated from above by a small 40-watt incandescent lamp. In response to the light, the animal would generally shelter its disk under the crevice and quickly retract its arms, forming multiple arm loops. When the animal had arranged itself into a stable configuration of coiled arms (after 1–5 min), the camera shutter was fired, triggering the strobes. The incandescent light was then extinguished, the covering rock removed, and the animal allowed to roam freely in darkness for 2–3 min before another trial. Ten trials were recorded for each individual.

Locomotion: One ophiuroid would be gently dropped upside down in the center of the tank, exposed to a faint illumination from one side. The animal would right itself and, using rhythmic arm strokes, walk away from the

Table 1

Body disk diameters (± 1 mm) for *Discovery Bay* ophiuroids

	Species						
	<i>Ophiocoma echinata</i>	<i>Ophioderma cinereum</i>	<i>Ophioderma appressum</i>	<i>Ophionereis reticulata</i>	<i>Ophiothrix oerstedii</i>	<i>Ophiothrix suensoni</i>	<i>Ophiocoma pumila</i>
	19	14	18	13	9	10	13
	18	15	13	12	7	9	9
	16	20	14	11	7	9	
	19	15	12	9	7		
	18	17	15	11	6		
	17	14	11	10	6		
	20	21	12				
	18	20	13				
	17	18	13				
	19	14	16				
No. of individuals	10	10	10	6	6	3	2
Mean disk diam.	18	17	14	11	7	9	11

light source. By observing the repeated excursions of the arms, the experimenter could fire the shutter at the approximate moment of the power stroke, during maximum curvature of the arms. After a pause of 2–3 min in darkness, the trial was repeated, for a total of 10 trials per individual. This technique is more prone to error than photography when the animal is stationary, because capturing the moment of maximum arm curvature is not guaranteed. Ophiuroid locomotion is relatively slow, however, so shutter-release errors should account for only small variations in the recorded position of the arm. Because all animals were photographed by the same experimenter (E.E.L.) under the same conditions, the tendency to err with this technique is not expected to bias comparisons among species.

Table II

Species list and ecological information

Species	Vertebral ossicle type	Feeding mode
OPHIOCOMIDAE		
<i>Ophiocoma echinata</i>	non-keeled	deposit-/suspension-feeder
<i>Ophiocoma pumila</i>	non-keeled	deposit-/suspension-feeder
<i>Ophiocoma wendtii</i>	non-keeled	deposit-/suspension-feeder
OPHIODERMATIDAE		
<i>Ophioderma appressum</i>	non-keeled	arm-loop scavenger
<i>Ophioderma cinereum</i>	non-keeled	arm-loop scavenger
OPHIOCHITONIDAE		
<i>Ophionereis reticulata</i>	keeled	deposit-feeder
OPHIOTHRAXIDAE		
<i>Ophiothrix oerstedii</i>	keeled	suspension-feeder
<i>Ophiothrix suensoni</i>	keeled	suspension-feeder

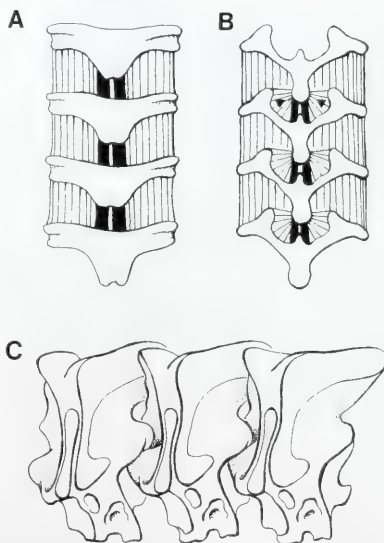


Figure 4. Dorsal views of vertebral series with external plates removed. (A) General arrangement of non-keeled vertebral ossicles (white), intervertebral muscles (striped), and dorsal ligaments (black). (B) Keeled vertebral ossicles form a nested series due to prominent aboral projections and depressions, connected by "accessory aboral muscles" (black triangles; Robbins, 1986). (C) Lateral view of a keeled vertebral series, showing overlap of adjacent ossicles within the arm.

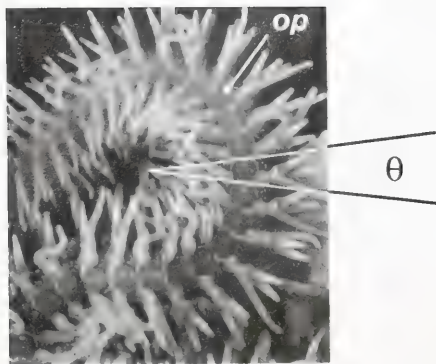


Figure 5. Enlarged photonegative of oral arm surface, *Ophiocoma echinata*. Borders of adjacent oral arm plates were used to measure intersegmental angles (θ). op = oral plate.

Digitizing technique

To quantify the degree of intersegmental rotation, more than 1300 photographic negatives were digitized using BioScan OPTIMAS software (ver. 3.1). The portions of the arm with the sharpest lateral curvature were visually selected from each frame. In the locomotion experiments, the region of sharpest curvature was selected from each arm participating in the locomotory "stride." These regions were generally in the middle third of the arm, where the segments were sharply bent to push against the substrate. In the coiling experiments, the region of sharpest curvature was selected from the proximal or middle portion of each arm that appeared tightly coiled. In this treatment, there were often several tight bends per arm, and most (but not necessarily all) of the arms were involved in this form of bending.

From each selected region, line segments were digitized along the proximal borders of 10 adjacent oral plates, and the angles between adjacent plates were calculated (Fig. 5). Oral plate borders were used as landmarks for comparing segment rotations because these boundaries appeared sharpest on the photographic negatives. Because the oral arm plates are not rigidly connected to the vertebral ossicle series, we use the term "intersegmental angles" to refer to these data. Although we did not visualize the vertebral series directly, in adjacent segments the oral intersegmental angle and the underlying intervertebral angle are probably quite similar, as both series of ossicles must follow essentially the same overall arm curvature. Even if oral arm plates are free

to move independently of the vertebral series, the angle between adjacent oral plates cannot consistently be larger (or smaller) than the underlying intervertebral angle, else the oral and intervertebral series would quickly diverge. Any offset between these two series of ossicles is thus likely to be small, and unlikely to introduce any persistent bias in comparisons between species. Thus intersegmental angles, measured externally from adjacent oral plate boundaries, are probably good estimators of intervertebral rotations *in vivo*.

For each series of 10 oral plate boundaries, the digitizing program automatically calculated all intersegmental angles (θ) and the maximum intersegmental angle achieved in that region ($\theta_{\max}$). When the segments are aligned along the proximal-distal axis, θ has a value of zero; it increases to a maximum as the segments rotate.

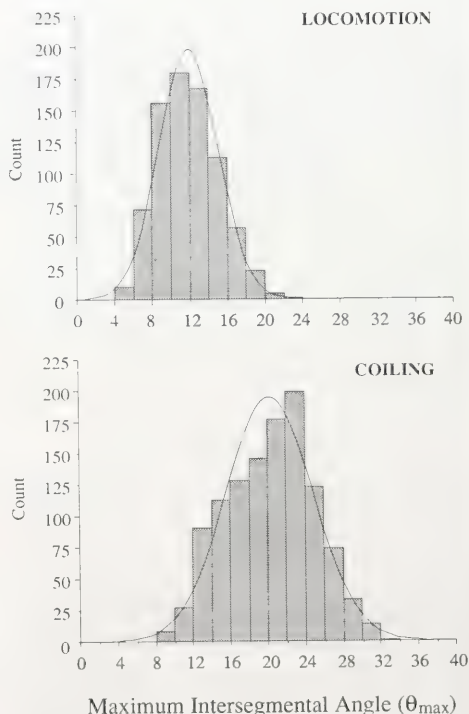


Figure 6. Histograms of maximum intersegmental angle ($\theta_{\max}$) for the locomotion and coiling experiments. Each histogram represents observations pooled over all species and trials. Note that the range of rotations in forced coiling is shifted upwards from the range observed for unrestrained locomotion.

The metric of interest is $\theta_{\max}$, because we wish to know whether different ophiuroid species are differently limited in their ability to flex between segments. Measurement error was determined by repetitive digitizing and was estimated to be $\pm 2^\circ$ ($n = 900$, $SD = 2.1^\circ$).

Results

Results from this photographic survey allow interspecific comparisons of lateral flexibility between ophiuroid arm segments. These data do not address other plausible planes of rotation, such as oral-aboral flexibility or torsion. Figure 6 compares maximum intersegmental angles pooled separately from all of the locomotion and coiling experiments. The largest intersegmental rota-

tions were observed during coiling trials (Fig. 6B), where both the minimum and the maximum observations exceeded those seen in unrestrained locomotion. This suggests that the direct illumination and limited refuge size used to elicit coiling prompts an increase in joint flexion. Because the observed rotations depend on the experimental conditions, data from the locomotion and coiling trials were analyzed separately.

Locomotion trials

Six species provided sufficient data for analysis of flexion during locomotion. Individuals within species showed similar means and variances for intersegmental angles, so all measurements from conspecifics were

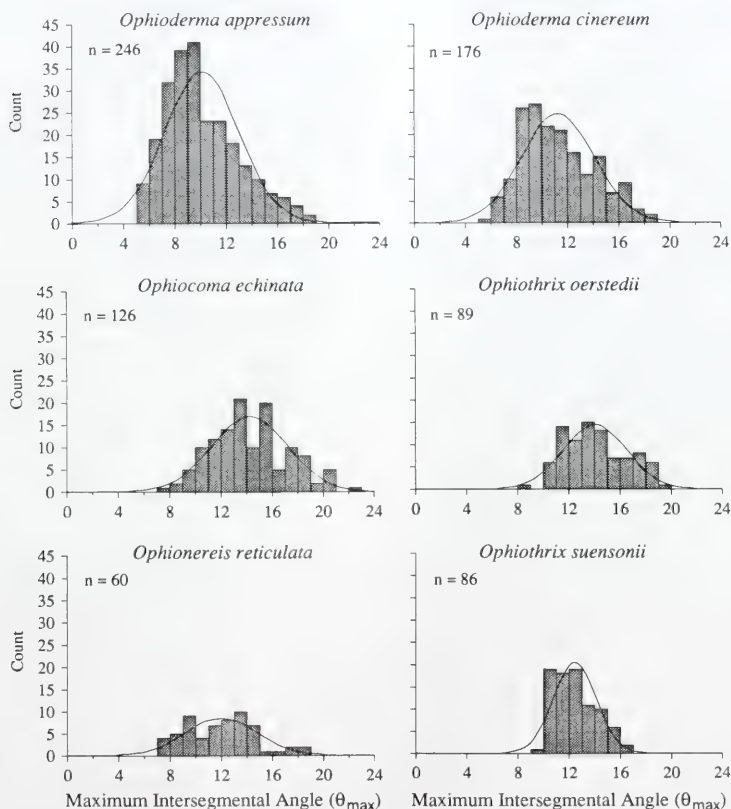


Figure 7. Histograms of maximum intersegmental angle ($\theta_{\max}$) for six Discovery Bay species photographed during free locomotion. n = number of maximal angles measured.

Table III

Mean maximum intersegmental angles (θ) and descriptive statistics for angular measurements on Discovery Bay species

Species	Mean ($^{\circ}$)	Std. Dev.	Std. Error	Min ($^{\circ}$)	Max ($^{\circ}$)	n
Locomotion trials						
<i>Ophioderma appressum</i>	10.2	2.9	0.18	5.3	18.3	246
<i>Ophioderma cinereum</i>	11.3	2.8	0.22	5.5	18.2	176
<i>Ophiocoma echinata</i>	14.3	2.9	0.26	7.3	22.3	126
<i>Ophiothrix oerstedii</i>	14.1	2.5	0.26	9.0	19.2	89
<i>Ophionereis reticulata</i>	12.0	2.8	0.36	7.6	18.9	60
<i>Ophiothrix suensonii</i>	12.5	1.6	0.17	9.9	16.0	86
Coiling trials						
<i>Ophioderma appressum</i>	17.5	3.8	0.29	9.4	27.9	168
<i>Ophioderma cinereum</i>	16.4	3.2	0.24	8.5	23.7	185
<i>Ophiocoma echinata</i>	22.1	3.1	0.19	14.4	29.4	259
<i>Ophiothrix oerstedii</i>	19.3	4.4	0.50	10.2	29.6	76
<i>Ophiocoma pumila</i>	23.1	3.0	0.34	15.9	29.8	78
<i>Ophionereis reticulata</i>	23.6	3.3	0.19	15.5	32.4	293
<i>Ophiothrix suensonii</i>	13.8	2.5	0.31	9.4	20.8	63

pooled. The resulting histograms of maximum intersegmental angles are shown in Figure 7. Note that each species exhibits a wide range of maximum angles in lateral bending, and that among species there is considerable overlap in the range of maximum angles measured. There are small interspecific differences in the mean maximum angle, or central tendency of each range. Because each distribution closely approximates a normal curve (Kolmogorov-Smirnov tests, each $P < 0.05$), parametric statistics were used to describe and compare these distributions. For multiple *post hoc* tests of the data, we used the Bonferroni correction (Rice, 1988) to adjust the within-test P value required for statistical significance.

Table III lists basic statistics for the distribution of maximal angles in each species. In lateral flexion during locomotion, most species exhibit the same range of maximal intersegmental angles (10° – 15°) between arm segments. Five of the six species show no significant differences in variance (multiple F tests, each $P > 0.003$); the sixth species, *Ophiothrix suensonii*, is significantly less variable ($P < 0.0001$) than the others. *Ophioderma appressum* and *O. cinereum* have the lowest mean rotations (10° – 11°). *Ophionereis reticulata* and *Ophiothrix suensonii* are intermediate ($\sim 12^{\circ}$), and *Ophiocoma echinata* and *Ophiothrix oerstedii* show the highest mean rotations ($\sim 14^{\circ}$). Figure 9A illustrates these differences by showing the species ranked according to mean maximum angle and grouped according to significant differences among means; each box encloses taxa whose means are not statistically different (multiple t tests, $P > 0.003$). Although these results show some significant interspecific differences in mean maximal rotation, the

absolute differences in this measure are small, and the functional significance of such differences is uncertain.

Coiling trials

Seven ophiuroid species are represented in the analysis of flexion during coiling. As before, maximal angles within species were normally distributed, and measurements from conspecific individuals were pooled. Species histograms of maximum intersegmental angles during coiling are shown in Figure 8. Each species exhibits a wide range of maximal angles, and there is considerable interspecific overlap in the maximal angles measured.

Descriptive statistics for the coiling distributions are shown in Table III. The coiling experiments tend to elicit mean lateral rotations of about 14° to 23° between arm segments. *Ophiothrix suensonii* tends to have the least rotation between segments (13.8°), whereas *Ophionereis reticulata* tends to have the greatest ($\sim 24^{\circ}$). The congeners *Ophioderma appressum* and *O. cinereum* have similar mean intersegmental angles, as do *Ophiocoma echinata* and *O. pumila*. The two species of *Ophiothrix*, however, exhibit different responses; *O. oerstedii* has an intermediate mean rotation, but the mean rotation of *O. suensonii* is rather low. Again, some interspecific differences in mean maximum angle are statistically significant, as shown by a ranking of species (Fig. 9B). Note that this order of species, from the lowest mean maximum to the highest, differs considerably from the order observed in the locomotion experiments (Fig. 9A). Thus the observed flexion depends not only on the species, but on the circumstances used to provoke arm flexion.

Interspecific differences in mean lateral flexibility do not clearly correspond to differences in vertebral ossicle morphology or feeding ecology. In the locomotion experiments, *Ophiocoma echinata* (a non-keeled, suspension/deposit feeder) and *Ophiothrix oerstedii* (a keeled, obligate suspension-feeder) both share the highest mean intersegmental rotations observed (about 14°). *Ophionereis reticulata* (a keeled deposit-feeder) and *Ophiocoma pumila* (a non-keeled deposit/suspension-feeder) are the most flexible species in the coiling experiments; their mean rotations (23.1° and 23.5° , respectively) are not significantly different. Thus species of different sizes, ecologies, and arm construction can achieve comparable intersegmental rotations in both of the activities measured here.

Discussion

Although there are significant variations in mean intersegmental angles among Discovery Bay ophiuroids, the absolute angular differences are quite small, and the degree of lateral flexibility is not obviously correlated

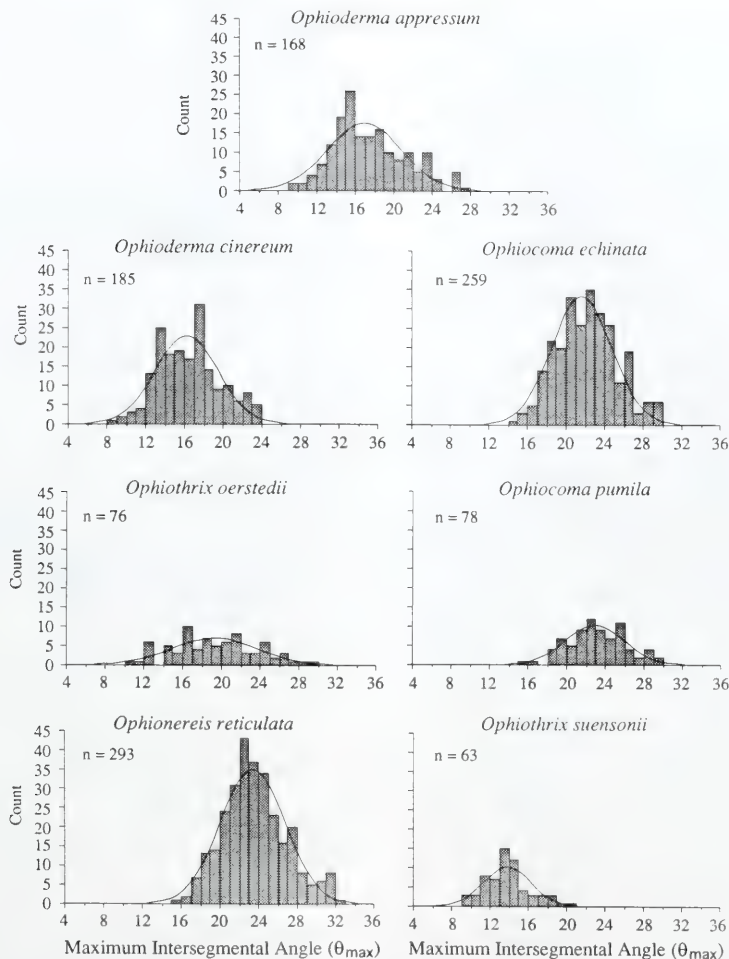


Figure 8. Histograms of maximum intersegmental angle ($\theta_{\max}$) for seven Discovery Bay species photographed while they were sheltering in a restricted space (= "coiling"). n = number of maximal angles measured.

with vertebral form or trophic mode. These data contrast with previous studies that have claimed mechanical significance for small interspecific variations in vertebral ossicle morphology. We tested this assumption by comparing species with non-keeled vertebral ossicles (*Ophiocoma*, *Ophioderma*), and species with keeled vertebral forms (*Opinionereis*, *Ophiothrix*). If these two vertebral types differ greatly in their capacity for lateral bending,

we might expect to see species from one group consistently out-bend those of the other.

The observed differences among ophiuroid species are more complex, as shown in a summary of the angular measurements (Fig. 10). First, the overall ranges of maximal rotations exhibited by different species greatly overlap. Second, a high mean maximal rotation is not confined to species of a particular vertebral type or feeding

strategy. Third, although the difference among means is significant for some species, the groups formed by this quantitative criterion do not clearly separate species according to either morphology or general ecology.

Species vary not only in their mean rotations, but also in the apparent extremes of rotation (Fig. 10). The largest single deflection between arm segments (32.4°) was observed in *Ophioneis reticulata* during coiling (Table III). This amount of lateral rotation is only slightly greater than that observed in *Ophiocoma pumila* (29.8°), *Ophiothrix oerstedii* (29.6°), and *Ophiocoma echinata* (29.4°) under the same conditions. Other species in this trial show much lower extremes, i.e., *Ophiothrix suensonii* (20.8°).

Though one might interpret these extreme values as species-specific "limits" on intersegmental flexibility, several caveats apply. (For a recent treatment of extremes in ecology, see Gaines and Denny, 1993.) As in any behavioral study, we can measure only the "actual" or observed performance of adjacent segments, not the "potential" performance of which they are theoretically capable. Actual intersegmental rotations clearly depend on experimental circumstances; nearly all species show greater rotations in the coiling trials, and some increase flexion more than others. *Ophiocoma pumila* and *Ophioneis reticulata* seemed particularly sensitive to this treatment, vigorously retracting their arms into multiple loops and

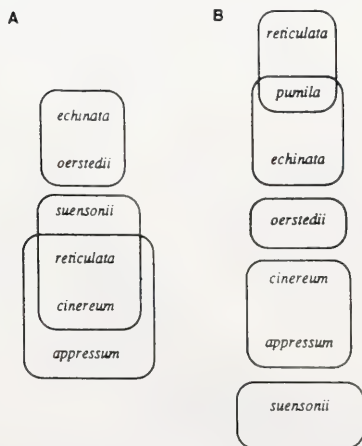


Figure 9. Graphical presentation of t test results. Species are ranked by mean maximum intersegmental angle ($\theta_{\max}$) from highest (at top) to lowest (at bottom). Each box encloses species whose means are not significantly different. (A) Locomotion trials, $P < 0.0033$. (B) Coiling trials, $P < 0.0023$.

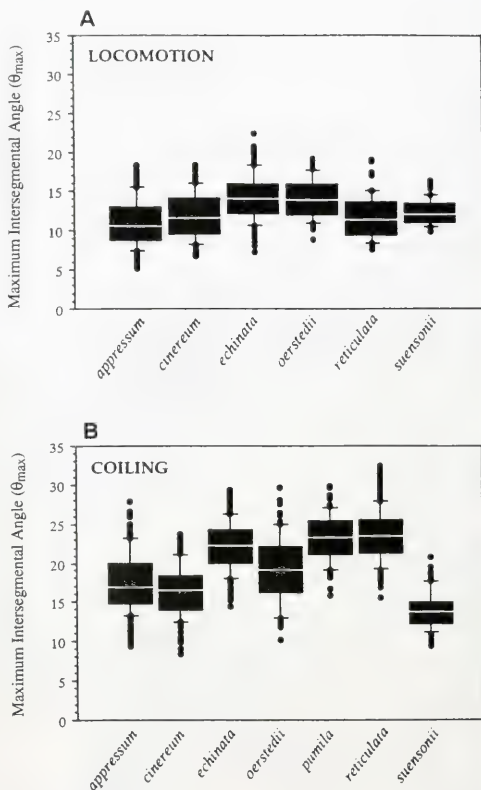


Figure 10. Boxplots comparing the range of maximum intersegmental angle ($\theta_{\max}$) for species in the locomotion (A) and coiling (B) experiments.

coils. The single species that is exposed during daylight (*Ophiothrix suensonii*) shows the least change between experimental treatments. These behavioral variations may confound comparisons of musculoskeletal performance, even when ophiuroids perform the "same" activities. For two species under the same conditions, actual bending may be close to the potential limit in one, but not in the other. Despite these limitations, our quantitative data suggest that there is nothing inherently limiting in either type of ophiuroid vertebral ossicle: within each treatment, species with keeled and non-keeled morphologies can achieve similar extremes of lateral rotation.

The lack of correspondence between vertebral form and maximal intersegmental angles in these species argues against the notion that vertebral morphology is a

dominant factor in limiting intersegmental rotation. As others have suggested, there is likely to be a complex interaction between the intervertebral articular surfaces, the external plates, and associated soft tissues in the ophiuroid arm (for review, see Byrne, 1994). Biomechanical influences on intersegmental rotation may include the extensibility of the integument (Byrne and Hendler, 1988), the neurally mediated stiffness of the connective tissue (Wilkie, 1978, 1979, 1992), the arrangement of the intervertebral musculature (Emson and Wilkie, 1982), and the conformation of the external arm plates (Litvinova, 1994). For example, Litvinova (1994) recognizes 15 types of ophiuroid arm structure on the basis of the size and shape of the external plates and their overall pattern of contact. These plate configurations are divided into three groups: those that "do not restrict," those that "slightly restrict," and those that "strongly restrict" arm movements. This classification, like most functional discussions of the ophiuroid vertebral system, seems to presuppose that there are inherent, species-specific differences in the capacity for intersegmental rotation.

Although we found no outstanding differences in intersegmental flexibility among the ophiuroids in our sample, this study is limited to the measurement of lateral flexion. The vertebral variants tested here may somehow contribute to interspecific differences in the range of dorsoventral bending or torsion. Until such differences can be measured, however, we must be more skeptical of the notion that vertebral ossicle morphology dictates flexibility between segments, and less speculative about how some types of segments are more (or less) flexible than others. If interspecific variation in intersegmental flexibility is more apparent than real, the present functional diversity of the Ophiuroidea cannot be explained solely by the evolutionary accumulation of vertebral ossicle variations that modify joint biomechanics. The ecological breadth of ophiuroids may be due to many combined factors of arm construction, including segment size; segment number; the control of muscles and mutable collagenous tissues; and the behavioral coordination of accessory spines, scales, and tube feet. The tendency to link vertebral variation with higher-level ecology in these animals is also at odds with several vivid accounts of how species can be ecologically labile, facultatively assuming three or four feeding modes (Fontaine, 1965; Fell, 1966; Reese, 1966; Magnus, 1967; Pentreath, 1970; Woodley, 1975; Feder, 1981). An alternative hypothesis is that vertebral ossicle morphology is variable because it has little or no influence on intersegmental rotation, and does not affect how (or how well) the ophiuroid makes its living.

Diversity in the external aspect of the skeleton has been the foundation of ophiuroid taxonomy. Diversity

in the internal skeleton remains poorly understood both in its morphological variance and in its functional effects. Although keeled and non-keeled ossicles are the major vertebral variants in these epifaunal species, other ophiuroid families show even more diverse arm morphologies and lifestyles. These include infaunal amphipods (which have extremely long, slender arms), epifaunal hemieuryalids (which have highly flexible, coiled grasping arms with a streptospondylous articulation), and the enigmatic ophiomyxids (which have a reduced axial skeleton and large amounts of connective tissue; see Byrne and Hendler, 1988). These groups are open for the further testing of form-function relationships.

Although the ophiuroid axial skeleton is conspicuously variable in morphology and obviously functional in its anatomical role, at the mechanical level we know little about how morphological variations influence intersegmental flexibility. At the ecological level, we lack convincing arguments to link flexibility *per se* to the style or success of an ophiuroid's locomotory or food-gathering movements. Further work must establish what are the differences in intersegmental flexibility, if any, among ophiuroid species, and in what way these differences are meaningfully correlated with ecologically relevant behaviors. More detailed behavioral visualization, mechanical testing of individual arm segments, and physiological recording of muscle activity may further elucidate the range and response of the intersegmental junction. Future discussions of the vertebral ossicle system should also make clear whether physical capabilities or biological roles are implied when skeletal morphology is described in functional terms (Gans and Gasc, 1992). Although ophiuroids are "flexible in feeding habits" (Fontaine, 1965) as well as flexible in motion, these two flexibilities may be only distantly related.

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Reduction of Growth Rate as the Major Process in the Miniaturization of the Sand Dollar *Sinaechinocyamus mai*

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Abstract. *Sinaechinocyamus mai* is an extremely small sand dollar, the maximum size being 10.9 mm. It has been suggested that *Sinaechinocyamus* is a miniaturized progenetic sand dollar that closely resembles the juveniles of *Scaphechinus*. In this study, we investigated the mechanisms responsible for the miniaturization. Our analysis of population dynamics, maturity, and annual reproductive cycles suggests that the growth rates of *S. mai* are about 19% the growth rates of *Scaphechinus mirabilis*, which reaches a maximum size of 88 mm. The developmental stages of oral and aboral surfaces were defined on the basis of the number of discontinuous interambulacral plates and the number of tube-foot porepairs, respectively. The patterns of the oral and aboral surfaces of the two species were compared, both at original size and after the *Scaphechinus mirabilis* pattern had been reduced to a size proportional to that of *S. mai* (i.e., to 19% original). On the oral surface, the patterns were different at the original sizes, but similar when the proportional sizes were compared; this indicates that the development of the oral plates is age-dependent in *S. mai*. On the aboral surface, the patterns were similar at the original sizes, but different in the proportional comparison, indicating that the development of the aboral plates is size-dependent in *S. mai*. *S. mai* becomes sexually mature at the age of 2 years, and *Scaphechinus mirabilis* matures probably at about the same age. Our data suggest that the reduction of growth rate (neoteny) is a more important mechanism of miniaturization in *S. mai* than is precocious cessation (progenesis).

Introduction

Miniaturization is the evolution of small adult body size and is a widespread phenomenon in animals (for re-

view, see Hanken and Wake, 1993). The developmental mechanism that mediates evolutionary change in body size and form has been addressed in a number of studies in terms of heterochrony (McKinney and McNamara, 1991). Changes in onset timing, offset timing, and growth rate in the entire individual (global heterochrony) or only in different characteristics but not throughout the individual (dissociated heterochrony), or even in opposite processes (mosaic heterochrony) have diversified body size and form (Skelton, 1993).

The direct mechanisms that result in miniature body size are the precocious cessation of growth, known as progenesis, and the reduction of growth rate, known as neoteny. Precocious cessation of growth means that the ancestral patterns of growth in mass and allometry are unchanged, but the duration of growth is truncated. Thus, lifespan is reduced by truncating growth and development, the reproductive system becomes mature precociously, and all developmental stages remain in the juvenile form of the ancestor. Reduction of growth rate means that the ancestral rate of growth in mass is reduced, but the pattern of allometry and duration of growth remains unchanged. Thus, all developmental and maturation rates remain consistent with those of the ancestor. Although the morphological characteristics are present at the expected age, they appear on a smaller scale as a result of the reduction in growth rate. The descendant is thus a microcosm of its ancestor. Indeed, different characteristics may have different heterochronic processes—the mechanism of miniaturization may not be simple at all.

Sinaechinocyamus mai, an endemic species in Taiwan, is an extremely small sand dollar, the maximum size being 10.9 mm in over 2000 individuals collected. It has an annual reproductive cycle, spawns from October to November, and has typical larval development (Chen

and Chen, 1993). In addition, it has intestinal diverticular sand grains that function as weight belts (Chen and Chen, 1994; Mooi and Chen, 1996). Mooi (1990) has suggested that *Sinaechinocyamus* is a highly derived, extremely miniaturized progenetic sand dollar that closely resembles juveniles of *Scaphechinus* in its patterns of oral and aboral plates, fossil record, and geographic distribution. *Scaphechinus mirabilis* is distributed in the waters surrounding Japan, and its maximum size may be 88 mm in transverse diameter (Nisiyama, 1968).

To reveal the mechanisms of miniaturization in *S. mai*, some ontogenetic data, including age, size, growth rate, developmental stages, developmental rate, and mature age, were collected. Generally, age is estimated by growth zones, or rings (Pearse and Pearse, 1975; Gage, 1991), but growth zones are not easily observed in *S. mai*. Thus, the age and growth rate were estimated according to the population dynamics, size at maturity, and annual reproductive cycle. Developmental characteristics include the separation of interambulacral plates on the oral surface, and an increase in the number of tube-foot pore-pairs in the petaloids. Therefore, the developmental stages of oral plates and aboral plates were determined as the number of discontinuous interambulacral plates and the number of tube-foot pore-pairs, respectively. The results reveal that the reduction of growth rate in *S. mai* is a more important mechanism for miniaturization than is precocious cessation.

Materials and Methods

Population dynamics and maturity

From October 1994 to March 1996, individuals of *Sinaechinocyamus mai* were periodically sieved with a 0.5-mm-mesh screen from sand flats in the low tide zone of Tunghsiao (120° 39' 45" E; 24° 29' 21" N), Miaoli, western Taiwan. The sizes of individuals collected were measured as the longest length through the anus.

Spawning was induced in individuals collected during the spawning season (October 1994 and 1995) by injecting 0.1 ml 0.5 M KCl into the coelom through the anus. Eggs were then fertilized artificially, and cleavage was verified with a Wild M3Z dissecting microscope. In addition, the gonads of individuals that did not spawn after injection with KCl were removed to determine their maturity. The gonads were fixed in 10% neutral formalin overnight, and then transferred to 70% ethanol, dissected by removing the oral half of the test, and examined with a dissecting microscope. The maturity of the gonads was determined using the methods of Chen and Chen (1993).

Growth rate analysis

According to Chen and Chen (1993), the spawning period of *S. mai* is from late October to early November,

so a spawning date of 1 November was used in estimating age. The growth curve was estimated by regression lines from each individual's size and its estimated age (month). The growth rate of *S. mai* was compared with that of *Scaphechinus mirabilis* as reported by Brykov and Parasyna (1979).

Development of oral plates

Tests were collected from the seashore in western Taiwan (including Hsinchu, Tainan, and Tunghsiao). The outlines of oral plates were drawn from tests placed under a Wild M3Z dissecting microscope equipped with a Wild TYP 308700 drawing tube. The visibility of the plates was increased by adjusting the intensity and incidence of the light. Tests less than 3 mm in length could not be studied under the dissecting microscope; therefore they were dried and coated, and then examined with a scanning electron microscope.

The developmental stage of the oral plates was determined by counting the discontinuous interambulacral plates. Each distinctly separate interambulacral plate was counted as 1 point. Plates in the process of separating (*i.e.*, not easy to distinguish whether separated or not) were counted as 0.5 point. Each sand dollar has 10 interambulacral plates, so the score for a test was derived by totaling the number of discontinuous plates among the 10.

Development of aboral plates

To trace the developmental characteristics of aboral plates, individuals were first treated with house bleach to remove the spines. The numbers of gonadal pores and tube-foot pore-pairs were then counted with the aid of a dissecting microscope.

The oral and aboral developmental stages of *Scaphechinus mirabilis* were based on descriptions in other papers (Nisiyama, 1968; Mooi, 1990) and on specimens collected from Japan by Dr. M. Komatsu and Y. Kano. Actual-size sketches of *Scaphechinus mirabilis* were traced in pencil on thin paper covering the tests.

Results

Size-frequency distribution

Living sand dollars were collected at Tunghsiao nine times from October 1994 to March 1996. With the exception of the February 1995 collection containing only 5 individuals, the size distributions are shown in Figure 1. Two size groups clearly existed in the sand dollars gathered in August and October 1995, while the other collections contained a few larger individuals (Fig. 1). One sand dollar collected in October 1994 was 10.9 mm in length.

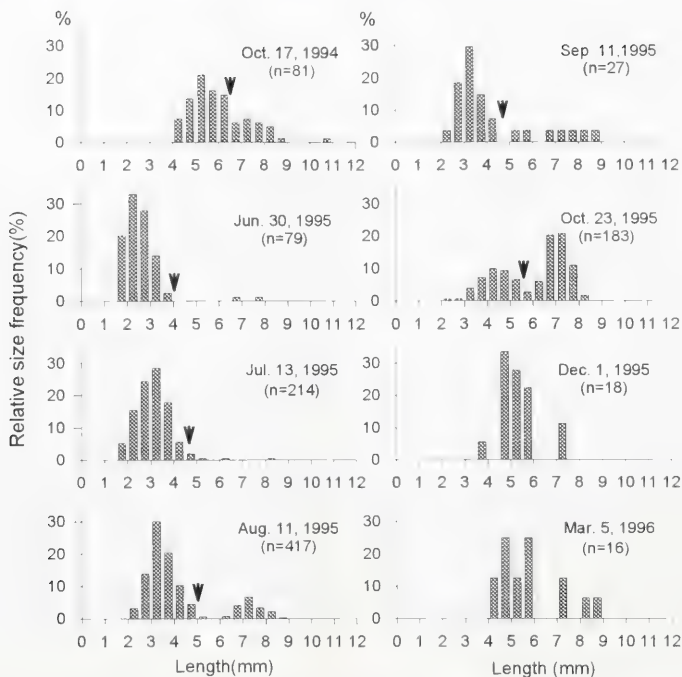


Figure 1. Size frequency distribution of *Sinachinocampus mai* from October 1994 to March 1996. Arrows indicate the division between two size groups on each graph except the last two, in which the number of specimens was not sufficient for groups to be discerned.

Maturity of gametes

Only large-sized individuals were able to spawn in response to injection of KCl solution during the spawning season. The 33 male and 32 female sand dollars examined during the spawning season showed no significant sex-related difference in size distribution. Individuals with mature oocytes or sperm were between 5.0 and 6.0 mm in length (Fig. 2).

The growth curve and comparison with *Scaphechinus mirabilis*

Since the two size groups of *S. mai* were distinct in degree of maturity, the groups were considered to represent different ages: specimens belonging to the small-size group were 1 year younger than their larger counterparts. The extra-large individual (10.9 mm in length) was considered to be a year older than the members of the large-size group.

This sand dollar spawns annually, therefore the 1995

small-size and large-size groups were assumed to be different age groups spawned in 1994 and 1993, respectively. Consequently, the growth rates of the first year and the second year were estimated from the age (in

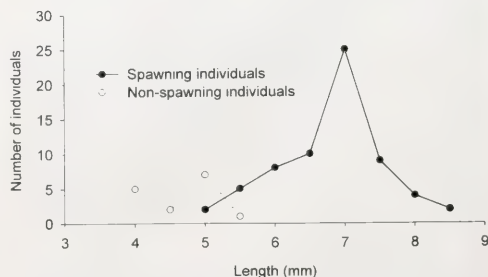


Figure 2. Size and maturity. Only large individuals are mature in spawning season.

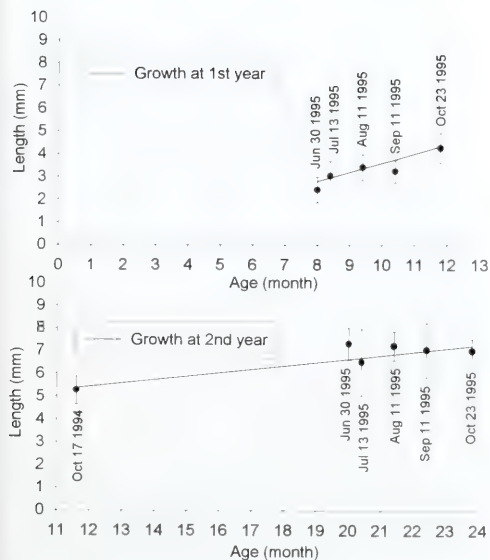


Figure 3. Linear regression between body length and age. Mean and standard error are shown for each age group.

months) and the length (in millimeters) of small-size and large-size groups, respectively. The regression equations are as follows:

$$Y_1 = -0.457 + 0.404(X_1),$$

$$(r = 0.307, t = 8.644, n = 720, P < 0.01)$$

$$Y_2 = 3.616 + 0.150(X_2),$$

$$(r = 0.544, t = 10.393, n = 259, P < 0.01)$$

From these two equations, the annual growth rate of the first year was estimated to be 4.4 mm/y ($Y_1(12)$), and that of the second year was estimated to be 1.8 mm/y ($Y_2(24) - Y_2(12)$) (Fig. 3).

The annual growth rates of *Scaphechinus mirabilis* in the first and second years are 23.4 and 9.2 mm/y, respectively (Brykov and Parasya, 1979). Therefore, the growth rates of that species in the first and second years are respectively 5.3 and 5.1 times faster than those of *S. mai*, and *Scaphechinus mirabilis* would be about 5.2 times larger than *S. mai* at the same age. Thus, the developmental rate of these two sand dollars should be compared by taking the actual-size pattern of *S. mai* and contrasting it with a reduced-size (to 19%) pattern of *Scaphechinus mirabilis*.

Developmental stages and rate of oral plates

Interambulacral plates separate in a specific order: posterior; posterior-right and posterior-left (whether posterior-right occurs second and posterior-left third or vice versa is still unclear); anterior-right; and anterior-left (Fig. 4A–F). Examination of the discontinuous interambulacral plates revealed that *S. mai* had a pattern different from that of *Scaphechinus mirabilis* when analysis was based on actual size. However, the full-size *S. mai* pattern was similar to the reduced-size *Scaphechinus mirabilis* pattern (Fig. 5). This indicates that development of oral plates is age-dependent.

One sample that was 8.3 mm in length had clear outlines of two rings on each plate (Fig. 6A, B). A model was constructed by assembling plates equivalent in size to the smaller of the rings on each plate of the 8-mm test. The resulting composite was similar to that of an individual 5.5 mm in length (Fig. 6B, C). The space between the rings indicated that the growth rates of plates in ambulatory were faster than in interambulacra. This allometric growth is identified as the process that causes separation of the interambulacral plates.

Developmental stages and rate of aboral plates

The number of tube-foot pore-pairs increased linearly with the increase in body length, from 8 pairs for a 1.5-mm body to 124 pairs for a 9.8-mm body. The number increased continuously and was not interrupted by the development of gonads and the formation of gonadal pores (Fig. 7). The increasing number of tube-foot pore-pairs showed that *S. mai* had a pattern similar to that of *Scaphechinus mirabilis* based on actual size, but dissim-

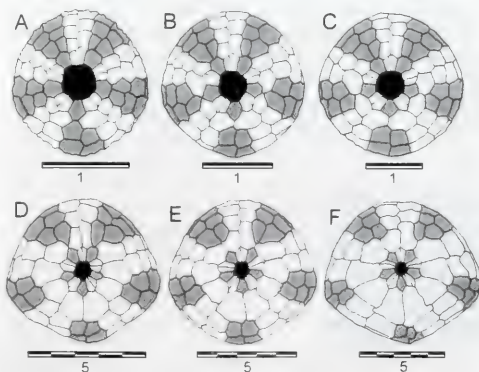


Figure 4. Developmental stage of oral plates. The interambulacral plates separate as size increases. Interambulacral plates are shaded; all scale bars are in millimeters.

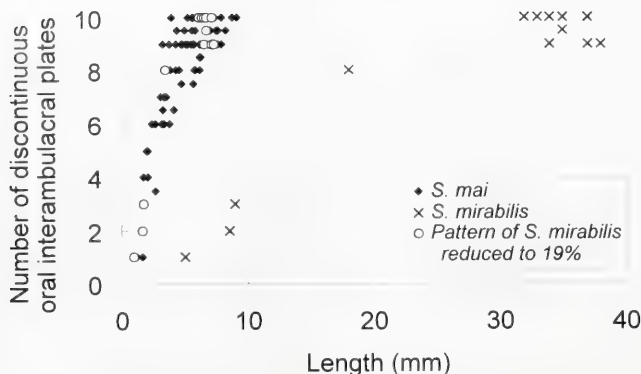


Figure 5. Developmental stages and developmental rate of oral plates.

ilar to the reduced *Scaphechinus mirabilis* pattern. That is to say, the increase in number of pore-pairs in *S. mai* was reduced by the same ratio as the reduction in growth rate.

Discussion

This study yielded several important results. First, the growth rates of *S. mai* are extremely slow: 4.4 mm/y in the first year and 1.8 mm/y in the second year. In *Scaphechinus mirabilis*, the corresponding rates are 23.4 mm/y and 9.2 mm/y (Brykov and Parasyina, 1979). The growth rate of *S. mai* is thus about five times lower than that of *Scaphechinus mirabilis*. Second, on the basis of size, the developmental stages of the oral plates (the increase in the number of discontinuous interambulacral plates) in *S. mai* is far "faster" than in *Scaphechinus mirabilis*, but the developmental stages of the aboral plates (the increase in the number of tube-foot pore-pairs) are almost the same in both sand dollars. However, age should be the basis for the comparison of developmental rates. When so compared, the developmental

rates of the oral plates are the same in both sand dollars, but the developmental rate of the aboral plates of *S. mai* is only 19% of that found in *Scaphechinus mirabilis*. Consequently, *S. mai* closely resembles juveniles of *Scaphechinus mirabilis*. Third, individuals of *S. mai* mature sexually (*i.e.*, have the ability to spawn) at the age of 2 years. Echinoids are typically 30% to 40% of their maximum size at first reproduction (Pearse and Cameron, 1991). Because the maximum size of *Scaphechinus mirabilis* is about 88 mm in length, the size of this species at first spawning is estimated to be 26.4–36.2 mm, which is larger than its 1-year-old size (23.4 mm), but includes its 2-year-old size (32.6 mm). These results suggest that the age of first spawning for *Scaphechinus mirabilis* would be between 2 and 3 years, close to the 2-year figure for *S. mai*.

As the growth rate of *S. mai* is five times slower than that of *Scaphechinus mirabilis*, there is no doubt that it has a tremendous effect on size. There is probably no difference between the ages of sexual maturity in *S. mai* and *Scaphechinus*. By inference, then, the mechanism for the miniaturization of *S. mai* is a reduction in growth rate. Thus, Mooi's suggestion of progenesis (1990) must be modified to neoteny. Since aboral and oral morphological characteristics have differences according to size, this miniaturized evolution is a dissociated heterochrony and the descendant, *S. mai*, is not a microcosm of its ancestor, *Scaphechinus mirabilis*.

Tube feet must attain a certain size in order to perform their normal function (Lawrence, 1987). Thus, the number of tube-foot pore-pairs located on the aboral plate is obviously dependent on the size of the plate. In *S. mai*, growth reduction keeps the aboral plates small, so only a few tube-foot pore-pairs can develop. Having established

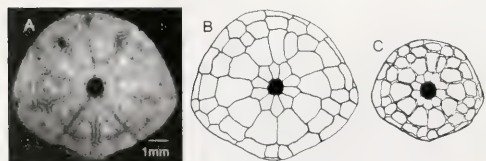


Figure 6. Original pattern of growth rings and the reassembly of first rings. The space between rings demonstrates that the growth rate in ambulacral plates is faster than in interambulacral plates. This allometric growth produced the separation of the interambulacral plates.

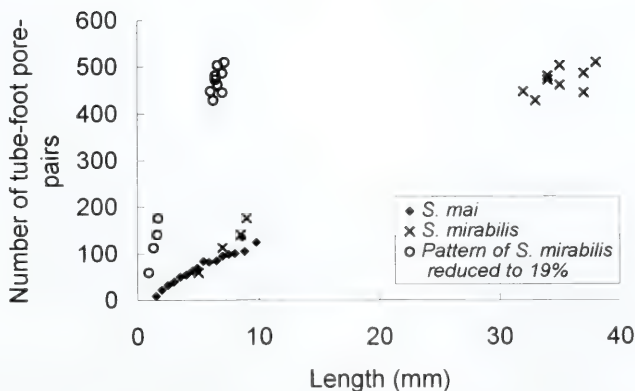


Figure 7. Developmental stage and developmental rate of aboral plates.

that this is a size-dependent characteristic, we propose that other characteristics responsible for the similarity of adult *S. mai* to juvenile *Scaphechinus mirabilis* may also be dependent on size. These would include aboral periprocts, simple internal partitions, and food grooves (listed by Mooi, 1990).

The development of oral plates in terms of the number of discontinuous interambulacral plates is a continuous process of plate rearrangement, and it is somewhat independent of size. Changes in onset, offset, and growth rate in different oral plates may cause different consequences; thus, the development of discontinuous plates becomes very variable in sand dollars (as in *Echinarchnius parma*; see Lohavanijaya and Swan, 1965). Comparisons of this characteristic should be limited to species that are closely related, like the ones in this study.

Sinaechinocyamus has existed in Taiwan since the Pliocene Epoch (Wang, 1984); *Scaphechinus* was recorded in the Pliocene Epoch in both Taiwan (Hayasaka and Morishita, 1947) and Japan (Mooi, 1989), but is extant in Japan only. Since both genera occurred simultaneously during the Pliocene, the initiation of speciation in *Sinaechinocyamus* is difficult to explain, but the success of miniaturization may be related to the presence of sand grains in the diverticula for position maintenance (Chen and Chen, 1994).

Mean global temperature dropped from the Miocene Epoch to the Quaternary Ice Age. Afterwards, the trend reversed, and global temperature reached its current state (Skelton, 1993). On the basis of water temperature changes and the geographical and stratigraphic occurrence of these two genera, we propose that *Sinaechinocyamus* might have evolved from a local, marginal population of *Scaphechinus* that extended to Taiwan from the

waters of Japan during the early Pliocene Epoch when the sea temperature was low. When water temperature rose, only miniaturized *Sinaechinocyamus* were able to continue their existence down to the present time in Taiwan. We hypothesize that the selective pressure on *Scaphechinus* was due to the warming sea temperature, which would increase metabolic rates, exhausting the materials and energy needed for maintenance and growth and leading to the disappearance of *Scaphechinus* from Taiwan. Conversely, a slow grower, even with the same metabolic rate, might have been able to tolerate the disorder caused by a warming of the water temperature. Thus, we speculate that the advantage of the small size of *Sinaechinocyamus* is related to the slow growth rate, which passively allows for existence in warmer waters.

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Identification and Localization of a Possible Rhodopsin in the Echinoderms *Asterias forbesi* (Asteroidea) and *Ophioderma brevispinum* (Ophiuroidea)

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Abstract. Protein extracts of optic cushion tissue from the asteroid *Asterias forbesi* and arm tissue from the ophiuroid *Ophioderma brevispinum* were subjected to Western blot analysis. Both tissues contain a membrane-associated protein that reacts with two monoclonal antibodies raised against bovine rhodopsin. This protein migrates slightly behind bovine rhodopsin during sodium-dodecyl-sulfate polyacrylamide gel electrophoresis, suggesting that its molecular weight is slightly larger. Immunohistochemical examination of the optic cushions of *A. forbesi* revealed a substance that reacts with both monoclonal antibodies; moreover, this substance is more abundant in dark-adapted animals than in light-adapted animals. The arms and central disk of *O. brevispinum* were also examined immunohistochemically. The tips of the arm spines contain a substance that reacts with both monoclonal antibodies, and at higher magnification this immunoreactive material is localized to small regions within the stroma of the ossicles. Taken together, the biochemical and immunochemical evidence suggests that the cross-reacting protein is homologous to other known rhodopsins and is serving as a visual pigment in echinoderms.

Introduction

Extraocular photosensitivity in echinoderms is widespread, well documented, and ecologically important (reviewed in Yoshida, 1979; Yoshida *et al.*, 1983), but the functional characteristics, morphology, and distribution of the receptors mediating this sense are poorly understood. At present, the location (subepithelial, dermal,

intra-ossicular, etc.) and ultrastructure (ciliary, rhabdomeric, unspecialized, etc.) of extraocular photoreceptors remain controversial (Cobb and Hendler, 1990; Cobb and Moore, 1986; Millott and Yoshida, 1960; Stubbs, 1981; Yoshida *et al.*, 1983). Among extraocular photoreceptors, those of ophiuroids are of particular interest because portions of the calcitic endoskeleton in ophiuroids transmit, polarize, and possibly focus light (Hendler and Byrne, 1987; Johnsen, 1994). In particular, the crystalline structure of the brachial spines of the ophiuroid *Ophioderma brevispinum* is ideal for analysis of the polarization of downwelling skylight (Johnsen, 1994). It has been shown that *O. brevispinum* is sensitive to polarized light (Johnsen, 1994), and it is hypothesized that this sensitivity is mediated by photoreceptors within the brachial spines.

If the visual pigment in echinoderms were identified, the photoreceptors could be localized using immunohistochemical techniques. The pigment is virtually unknown (reviewed in Yoshida *et al.*, 1983), but several assumptions can be made about it. First, the visual pigment is likely to be a rhodopsin, because all described visual pigments are members of this protein family. Second, it is assumed that essentially the same visual pigment occurs in the photoreceptors of all echinoderms. Third, it is assumed that the optic cushions of asteroids are functionally similar to other metazoan eyes, because a large body of chemical, morphological, and behavioral results suggests that optic cushions are visual organs (Takasu and Yoshida, 1983; reviewed in Yoshida *et al.*, 1983). Given these assumptions, the asteroid optic cushion can be used as a model structure for determining the visual pigment in echinoderms. The optic cushion of *Asterias forbesi* is particularly suitable as a model because

it is known that the microvilli of the putative sensory cells within the optic cup of a congener (*A. amurensis*) become more numerous and increase in size and retinal protein content after long exposure to darkness (Takasu and Yoshida, 1983).

This study describes an immunochemical search for homologues of rhodopsin, first in the optic cushion of the starfish *Asterias forbesi* and then in the tissues of the brittlestar *Ophioderma brevispinum*. In both animals, the search revealed a membrane-associated protein, slightly heavier than bovine rhodopsin, that reacts with two monoclonal antibodies raised against bovine rhodopsin. The same antibodies also react with a substance in the optic cups of the optic cushion of *A. forbesi*. In addition, the cross-reaction is stronger in dark-adapted animals than in light-adapted animals. The two monoclonal antibodies also cross-reacted with a substance within the tips of the brachial spines of *O. brevispinum*, suggesting that they contain concentrated amounts of echinoderm rhodopsin. These data all suggest that the purified protein in both animals is homologous to known rhodopsins and that this protein is the visual pigment in echinoderms.

Materials and Methods

Source and maintenance of animals

Specimens of *A. forbesi* were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. Specimens of *O. brevispinum* were collected from Old Dan Bank, about 2 km north of the Keys Marine Lab, Layton, Long Key, Florida. Animals were maintained in aquaria in artificial seawater (ASW), salinity 35 ppt, on a 12/12 hour light/dark cycle. *O. brevispinum* and *A. forbesi* were kept at 24°C and 10°C respectively.

Extraction and Western blot analysis

An adult specimen of *A. forbesi* was dark-adapted for about 20 h. The adaptation was performed because previous studies on several asteroids have shown that the microvilli of the putative sensory cells in the optic cushions increase in number, size, and retinal protein content after long exposure to darkness (Takasu and Yoshida, 1983). Under dim red light, the tips of four arms (containing optic cushions) were removed and immediately frozen in liquid nitrogen. The frozen tissue was pulverized with a mortar and pestle and the powder suspended in 100 μ l sodium-dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer. The solution was incubated at 20°C for 5 min to dissolve all proteins and centrifuged at $16,000 \times g$ for 10 min to precipitate insoluble material. Because rhodopsins that have been boiled in the presence of SDS

irreversibly aggregate, the samples were not boiled before electrophoresis. The supernatant was electrophoresed on an 8% SDS polyacrylamide gel and transferred to a polyvinyl membrane (Immobilion-P, Millipore Inc.) using the Western transfer process (Harlow and Lane, 1988). The gel lanes were loaded with the maximum amount of protein that could run correctly on the gel apparatus. Nonspecific binding was blocked by incubating the membrane in 1 μ g/ml polyvinyl alcohol for 5 min. The membrane was then incubated in a monoclonal antibody raised against bovine rhodopsin (diluted 1:50 in phosphate buffered saline (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4, referred to hereafter as PBS) containing 0.1% Tween-20 detergent and 3% bovine serum albumin (BSA)) for about 20 h at 20°C. Fourteen monoclonal antibodies raised against bovine rhodopsin were tested. Their epitope was then investigated using competitive binding assays with various peptide sequences from bovine rhodopsin (Adamus *et al.*, 1991). Figure 1 shows the names and putative epitopes of the antibodies. All 14 antibodies are known to bind with bovine rhodopsin on a Western blot (Hargrave, pers. comm.). Antibodies raised against bovine rhodopsin were chosen because echinoderms are more closely related to chordates than to any other phylum for which rhodopsin antibodies are available. After reaction with the primary antibody, the membrane was washed three times for 5 min each in PBS, and then incubated for 1 h at room temperature in goat anti-mouse IgG + IgM conjugated with alkaline phosphatase (Sigma Immunochemicals; diluted 1:5000 in same buffer as primary antibody). The membrane was then washed three times for 20 min each in PBS and developed according to standard procedures for alkaline phosphatase detection (Harlow and Lane, 1988). The procedure for *O. brevispinum* was identical except that tissue from the arms was used. As a negative control, tissue from the gut of *A. forbesi* was prepared and analyzed in the above manner.

Determination of cellular location of proteins from *A. forbesi* and *O. brevispinum*

Tissue from *A. forbesi* or *O. brevispinum* was frozen and pulverized as described above. The powder was suspended in 200 μ l of PBS with 1 mM phenylmethylsulfonyl fluoride and 20 μ g/ml leupeptin (referred to hereafter as PBS/PI). The solution was centrifuged for 20 min at $16,000 \times g$. The supernatant was then centrifuged at $125,000 \times g$ for 1 h to remove all insoluble material. The supernatant from the high-speed spin was kept and referred to as "C." The pellet from the low-speed spin was resuspended in 200 μ l of PBS/PI with 2% Triton X-100 and incubated for 30 min. The suspension was then spun

B6-30N

MNG TEG PNF YVP FSN KTG VVR SPF EYP QYY LAE PWQ YS LAA YMF LL Q YI
 R2-15N | K62-12N

NEW TLV VTV QHK KLR TPL NYI LLN LVF ANH FMV EGG FTV TMV TSL HGY FVF GPT
 B10cL + B3uL A1-55M

GCY IEG FFA TLG GE ALW SLV VLA LER YV CK PMA NFR FGE DHA MG V F IW

MAI ACA APP LAG WSR YIP EGM QCS CGV DYY TLK PEI NNE SFV YM F V HF P
 T13-34L + T13-35L

ILI PFC YG LI TVK EAA AQQ OES ATT OKA EKE VTR MVI IMV ME L Q W P YAI
 K42-41L

VAF YIF THQ GSN FGP VFM TIP APF AKS SAI YNP VIY IMM NKQ FRN CMV TTL CCG
 A11-275C

K16-50C

KNP LGD DEA STT ASK TET SOV APA
 K57-91C K16-155C + k55-75C

Figure 1. Amino acid sequence of bovine rhodopsin showing the epitopes of the various antibodies used in the study. Light type denotes transmembrane regions that are inaccessible to antibodies in immunohistochemistry. Bold letters indicate amino acid identity among the following 11 chordate rhodopsins: *Lampetra japonica*, *Carassius auratus*, *Xenopus laevis*, *Rana pipiens*, *Gallus gallus*, *Ovis aries*, *Bos taurus*, *Mus musculus*, *Homo sapiens*, *Cricetulus griseus*, and *Canis familiaris*. Underlined and overlined regions indicate the epitopes of the antibody listed by the line.

at $16,000 \times g$ for 20 min. The supernatant was kept and referred to as "M." Supernatant C (containing proteins soluble in physiological buffer) and supernatant M (containing proteins insoluble in physiological buffer, but soluble in a mild detergent) were analyzed using Western blot analysis.

Histology of optic cushions from *A. forbesi*

The distal tips of the arms of dark-adapted *A. forbesi* were excised and fixed overnight in darkness in 10% formalin buffered in artificial seawater. The tissue was de-

calcified in DeCal (National Diagnostics Inc., Manville, NJ), dehydrated in a graded series of ethanols, cleared in HistoClear (National Diagnostics Inc.), and embedded in paraffin. Serial sections $10 \mu\text{m}$ thick were stained with Picro-Ponceau and Weigert iron hematoxylin (Hudson, 1979). For additional details concerning histological methods, see Kier (1992). The sections were viewed by both light and confocal microscopy (Zeiss LSM 410 Invert, Thornwood, NY). Light microscopy was used to confirm the staining properties of the various elements, and confocal microscopy was used to increase depth resolution.

Immunohistochemistry of A. forbesi optic cushions and O. brevispinum arms and central disk

The following procedures were performed in either dim red light or total darkness. The dehydrations, hydrations, permeabilization, washes, and antibody incubations were done on a tissue rotator at 4°C. Animals were either light- or dark-adapted for 20 h. Tissue was fixed for 2 h at 20°C in 10% formalin in ASW, dehydrated using a graded series of ethanols (30%, 50%, 70%, for 1 h each), and incubated in 95% ethanol for 20 h to extract screening pigments. The tissue was then hydrated, permeabilized in 2% Triton X-100 in PBS for 20 h, and incubated in either B6-30N or R2-15N (diluted 1:50 in PBS/Tween with 3% BSA) for 2 days. The tissue was washed in PBS for 30 min and then incubated in donkey anti-mouse IgG conjugated with Cy5 or fluorescein (diluted 1:1000) for 1 day. Finally, the tissue was washed for 20 h in PBS and mounted in 300 µl of *n*-propyl-gallate. The stained tissue was observed and optically sectioned using a confocal microscope with accompanying software.

Results

Biochemical and immunochemical characteristics

Of the 14 monoclonal antibodies tested, two antibodies, B6-30N and R2-15N, cross-reacted with a protein found in both *A. forbesi* and *O. brevispinum*. None of the 12 other monoclonal antibodies cross-reacted with any proteins found in both animals. Lane BR of Figure 2 shows a Western blot of an extract of bovine rod outer segments incubated in B6-30N. The lanes marked C are Western blots showing the absence of the cross-reacting protein in a solution of proteins soluble in physiological buffer. The lanes marked M are Western blots showing the presence of the cross-reacting protein in a solution of proteins insoluble in a physiological buffer but soluble in Triton X-100. Both lanes C and M are replicated for *A. forbesi* and *O. brevispinum*. Tissue incubated both in B6-30N and in R2-15N and the results suggest that the cross-reacting protein is membrane-associated. The lanes also show that the mobility of the protein during SDS-PAGE is slightly less than that of bovine rhodopsin. Lane G shows a Western blot of the gut tissue from *A. forbesi* using the B6-30N showing that there is no cross-reaction.

Histology and immunohistochemistry of optic cushion

The morphology of the optic cushion and optic cups of *A. forbesi* is similar to that observed in *Marthasterias glacialis* and *Asterias amurensis* (Smith, 1937; Yoshida, 1966). The optic cushion is an oral, hemispherical expansion of the terminus of the radial nerve cord found directly below the terminal tentacle of the arm (Fig. 3A).

It contains approximately 50 evenly spaced, pigment-lined, conical optic cups and many isolated pigment spots. The pigment is an intense reddish orange. Figure 3B shows the morphology of a single optic cup. The mouth of the cup is covered by a 0.6-µm-thick cuticle and a lenticular layer (up to 5 µm thick) of optically homogeneous tissue that does not react with any of the stains used. The cup is lined with two types of cells. The first type is more-or-less cuboidal and contains many spherical pigment granules. This cell type is referred to here as a putative pigment cell, because it matches the morphology and distribution of the pigment cells described by Smith (1937) and Yoshida (1966). The second type of cell is elongated and originates from branching fibers with the staining characteristics of nerve tissue; it passes between the putative pigment cells and extends into the lumen of the optic cup. This cell type is referred to here as a putative sensory cell, because it matches the morphology and distribution of the sensory cells described by Smith (1937) and Yoshida (1966). The results of the immunohistochemistry of the optic cushions are shown in Figure 4. The optic cushion does not react with the secondary antibody (Fig. 4A). The B6-30N antibody cross-reacts with a substance located within the optic cups and the isolated pigment spots of the dark-adapted animals (Fig. 4B). The B6-30N antibody also cross-reacts with a substance located within the optic cups of light-adapted animals, but the cross-reaction is considerably weaker. Figure 4C shows the strongest cross-reaction observed in a light-adapted animal. No significant staining was observed in the spines or the tube feet. The above results were also obtained using the R2-15N antibody (not shown).

Immunohistochemistry of O. brevispinum

The results of the immunohistochemistry of *O. brevispinum* are shown in Figure 5. The B6-30N antibody cross-reacts consistently with a substance located within the distal tips of the arm spines. No reaction with the secondary antibody is seen in the control (Fig. 5A). The cross-reaction is stronger in dark-adapted animals than light-adapted animals (Fig. 5B, & C), though the difference is smaller than that observed in *A. forbesi*. The staining is stronger on the lateral spines than on the more oral or aboral spines. There is no consistent change in staining intensity between arm spines on proximal and distal arm segments. No consistent staining was found on any other portion of the arms or on the central disk, though occasional staining was observed at the bases of the arm spines, and a light staining was occasionally observed over the entire surface of the arms. The above results were also obtained using the R2-15N antibody (not shown).



Figure 2. Digitized photographs of Western blots. Lane BR: Western blot (using BL-30N) of solution of bovine rod outer segments. The two bands are the monomer and dimer of bovine rhodopsin. Lanes M: Western blot (using B6-30N or R2-15N) of proteins from *Asterias forbesi* or *Ophiasterias brevispinum* that are insoluble in a physiological buffer but soluble in Triton X-100 detergent. Lanes C: Western blot (using B6-30N or R2-15N) of proteins from *A. forbesi* or *O. brevispinum* that are soluble in a physiological buffer. Lane G: Western blot (using BL-30N) of gut tissue from *A. forbesi*.

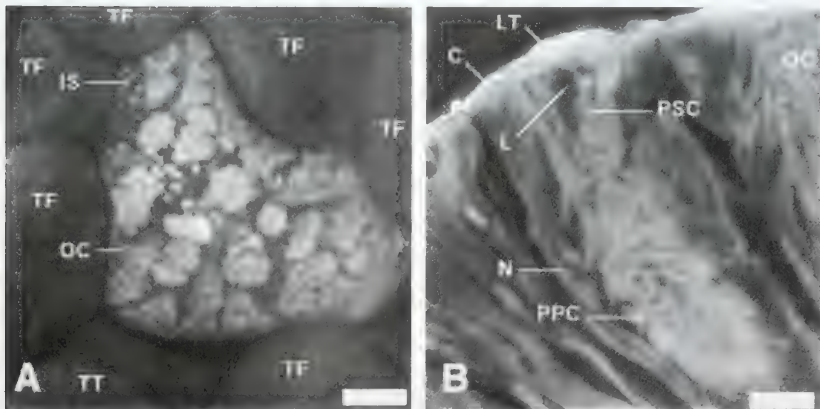


Figure 3. (A) Digitized image of oral view of the arm tips of *Asterias forbesi* obtained by overlaying 40 confocal images taken at focal depth intervals of 10 μ m. Red pigment is visualized as white. The proximal-distal axis of the arm runs from the upper-right corner to the lower-left corner. Scale bar is 50 μ m. (B) Digitized image of optic cup of *A. forbesi* obtained by overlaying 5 confocal images taken at focal depth intervals of 1 μ m. Images were taken from a 10- μ m paraffin section stained with Picro-Ponceau and Weigert iron hematoxylin. Scale bar is 10 μ m. OC, optic cup; IS, isolated pigment spot; C, cuticle; N, nerve; PPC, putative pigment cell; PSC, putative sensory cell; LT, lenticular thickening; TT, terminal tentacle; TF, tube foot; L, lumen of optic cup.

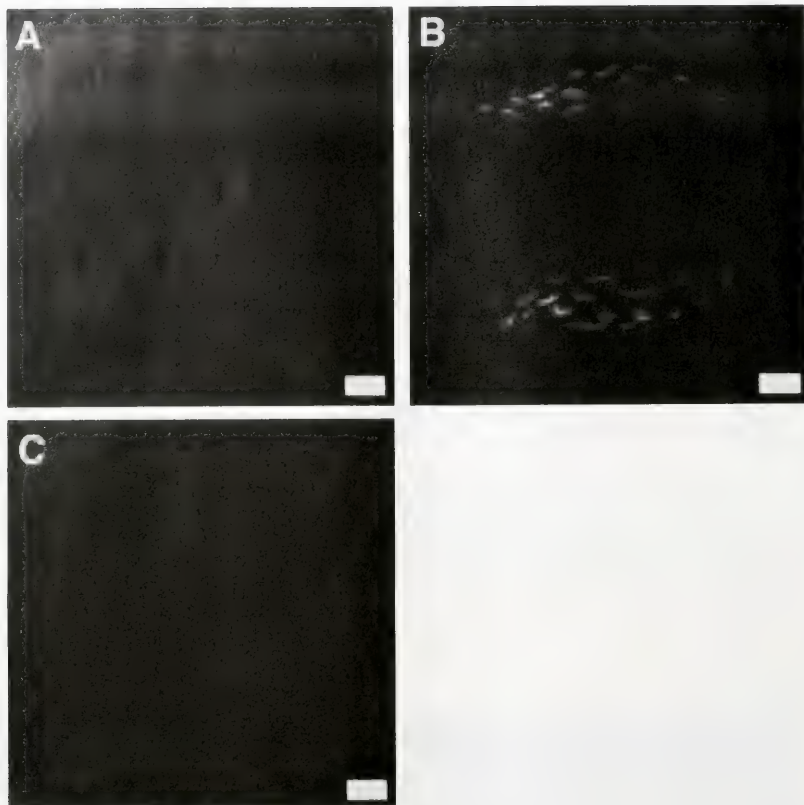


Figure 4. Digitized images of arm tips of *Asterias forbesi* obtained by overlaying 40 confocal images taken at focal depth intervals of 10 μm . (A) Control with dark-adapted animal incubated with antibody buffer followed by secondary antibody. (B) Dark-adapted animal incubated with B6-30N antibody followed by secondary antibody. (C) Light-adapted animal incubated with B6-30N antibody followed by secondary antibody. Brightness and contrast in A, B, and C are identical. Scale bar is 50 μm .

Discussion

Biochemical and immunochemical evidence relating the visual pigment in echinoderms to chordate rhodopsins

The Western blot analyses and immunohistochemistry provide four lines of evidence that the protein that cross-reacts with the two monoclonal antibodies is homologous to known rhodopsins. First, the mobility of the protein during SDS-PAGE approximates that of bovine rhodopsin. Second, the protein is insoluble in a physiological buffer but soluble in a mild detergent, suggesting that it is membrane-associated. Third, Western blot analysis shows that the purified protein cross-reacts

strongly and specifically with two monoclonal antibodies raised against bovine rhodopsin. Finally, both antibodies cross-react with a substance within the optic cups of *A. forbesi*, and this substance is more abundant in dark-adapted animals than in light-adapted animals. The morphology of the optic cushions and the optic cups of *A. forbesi* is similar to that found in other *Asterias* species that have been studied. Ultrastructural and histofluorescence work on these congeners has demonstrated the presence of organized microvilli and retinal proteins within the lumen of the optic cups (Eakin and Brandenburger, 1979; Penn and Alexander, 1980; Takasu and Yoshida, 1983). In addition, histofluorescence and ultra-

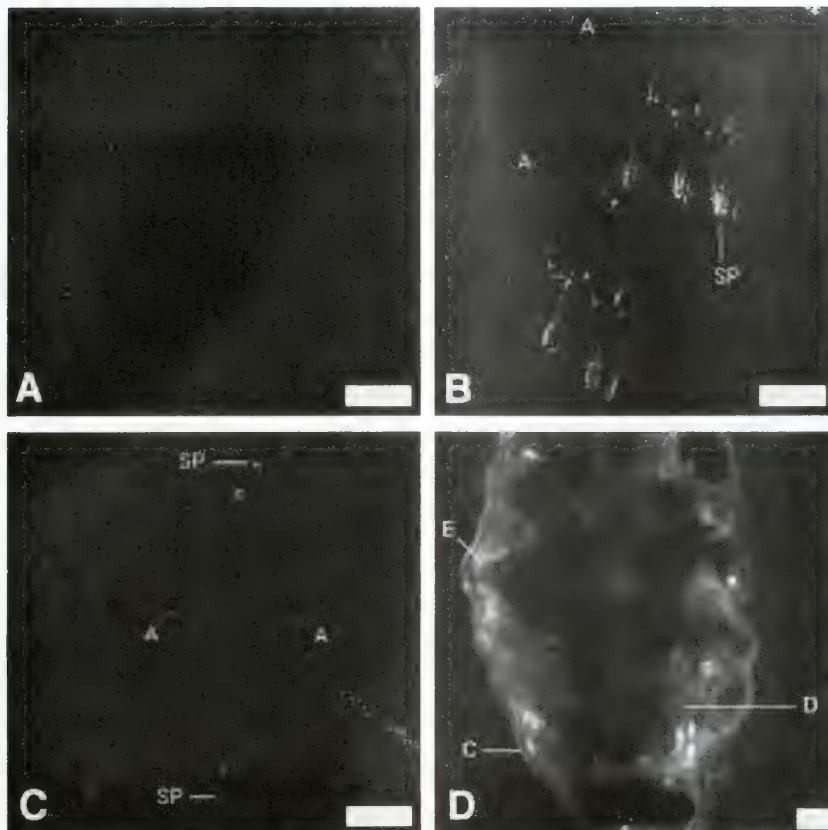


Figure 5. Digitized images of aboral views of 2-3 segments of arms of *Ophioderma brevispinum* obtained by overlaying 40 confocal images taken at focal depth intervals of 10 μm . Each segment has two sets of 7 lateral spines each (not all spines visible). (A) Control with dark-adapted animal incubated with antibody buffer followed by secondary antibody. (B) Dark-adapted animal incubated with B6-30N antibody followed by secondary antibody. (C) Light-adapted animal incubated with B6-30N antibody followed by secondary antibody. Brightness and contrast in A, B, and C are identical. Scale bar is 200 μm . (D) Digitized image of a single longitudinal section of the tip of the central arm spine in B. Scale bar is 10 μm . SP, arm spine; A, aboral arm plate; C, cuticle; E, epidermis; D, dermis.

structural investigations have shown that these microvilli and retinal proteins are more abundant in dark-adapted animals than in light-adapted animals (Takasu and Yoshida, 1983). Therefore, the substance in the lumen of the optic cups that cross-reacts with the two monoclonal antibodies is probably a rhodopsin and thus, given the specificity of the antibodies in these animals, the cross-reacting protein found in the Western blots.

The possibility remains, however, that the two monoclonal antibodies are reacting specifically with a protein

that is unrelated to photoreception. There are two arguments against this possibility. The first is based on the biochemical characteristics of the cross-reacting protein—if the protein is not rhodopsin, it is a membrane-associated protein similar in size to vertebrate rhodopsins. The second involves location—the immunochemistry suggests that the cross-reacting protein is strongly concentrated in the optic cups of *A. forbesi* and more common in dark-adapted than light-adapted animals, suggesting that the protein is involved in echinoderm

photoreception. The biochemical and immunochemical characteristics considerably limit the number of possible cross-reacting proteins. However, despite the above evidence, protein sequencing or ultrastructural examination of the cross-reacting tissues is required before definite conclusions can be drawn.

Immunohistochemistry of O. brevispinum

The immunohistochemistry of *O. brevispinum* shows that a substance within the tips of the arm spines cross-reacts with monoclonal antibodies raised against bovine rhodopsin. This, in conjunction with the biochemical evidence, the specificity of the antibody, and the immunohistochemistry of the optic cushions of *A. forbesi*, suggests that the substance within the spine tips is a rhodopsin. This result is significant because a variety of ciliary and microvillous structures, presumed sensory in nature, are concentrated at the tips of ophiuroid arm spines (Whitfield and Emson, 1983; Cobb and Moore, 1986). If the photoreceptors of *O. brevispinum* are also concentrated in these regions, then the arm spine tips may be the primary sensory regions of the animal, similar to rhopalium in scyphozoans or asthaetes in polyplacophorans.

This result is also significant because previous work has shown that *O. brevispinum* has polarization sensitivity and that the ossicles of its endoskeleton analyze polarized light (Johnsen, 1994). It is hypothesized that the animal's polarization sensitivity is mediated by differential transmission through its polarizing skeleton. The arm spines are the only ossicles correctly oriented for detecting the plane of polarization of overhead light, so it is appropriate that these ossicles contain photoreceptors. The middle, most lateral, arm spines project horizontally from the lateral surface of the arm, and thus are precisely oriented for detecting overhead polarized light. That they also contain the most rhodopsin (see *Results*) is, therefore, intriguing.

The possible localization of rhodopsin within the tips of the arm spines does not preclude the presence of rhodopsin in other portions of the animal. It is likely that the cuticle and calcitic endoskeleton limited penetration of the antibodies. In addition, the frequent interfaces between the calcitic endoskeleton and the organic matrix scattered light, thus limiting the ability of the confocal microscope to obtain high-resolution images at a depth of more than 25 μm into the ossicles. Electrophysiological evidence has shown that the oral and aboral arm plates of *Ophiocoma wendtii* and *Ophiura ophiura* are sensitive to light (Moore and Cobb, 1985; Cobb and Hendler, 1990). In the case of *O. wendtii*, the photoreceptive regions were found to lie quite deep within the ossicles. If these regions exist in *O. brevispinum*, they

may be beyond the limit of antibody penetration or coherent imaging by the confocal microscope. Attempts to circumvent these limitations by staining sectioned material were unsuccessful, possibly due to deterioration of the epitope by the embedding procedures (Brandtzaeg, 1982).

In summary, this study identifies a possible visual pigment in the echinoderms. The pigment appears to be a rhodopsin with a molecular weight slightly larger than that of chordate rhodopsins. Immunohistochemistry of the brittlestar *O. brevispinum* shows that this pigment is concentrated within the tips of the arm spines, suggesting that these regions are photoreceptive. The presence of photoreceptors in the spine tips is interesting, both because many sensory cells in ophiuroids are found there and because the polarization properties of the spine ossicles may explain the polarization sensitivity of the animal.

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**Marine
Biological
Laboratory
Woods Hole
Massachusetts**

**Ninety-Ninth Report
for the Year 1996
One-Hundred and Eighth Year**

Officers of the Corporation

Sheldon J. Segal, *Chairman of the Board of Trustees*

Frederick Bay, *Co-Vice Chair*

Mary J. Greer, *Co-Vice Chair*

James D. Ebert, *President of the Corporation*

John E. Burris, *Director and Chief Executive Officer*

Mary B. Conrad, *Treasurer*

Neil Jacobs, *Clerk of the Corporation*

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Report of the Director and Chief Executive Officer

1996 was a year of great excitement at the Marine Biological Laboratory, not only for our accomplishments in science and education, but also for the Trustees' decision to undertake a comprehensive fundraising campaign which will ensure that the MBL is a strong research and educational institution in the 21st Century. This campaign has as its goal to raise \$25 million by the end of the year 2000 in support of research, education, and facilities.

Biological research does not stand still, and neither can an institution devoted to its progress. As the 20th Century becomes the 21st, the world needs to be able to count on science and technology—and the MBL—to help confront the challenges of curing disease, feeding the hungry, keeping the environment healthy, and improving the overall quality of peoples' lives.

Thanks to careful planning, the MBL is now poised to meet these challenges. A successful campaign will enable us to establish new research centers of excellence, create and build an endowment for our educational program, enhance research opportunities for our summer and visiting investigators, and improve the facilities that support these programs.

The Campaign for the Marine Biological Laboratory will enable the Laboratory to enter the 21st Century as a secure, independent center of biological, biomedical, and environmental research and education, positioned to undertake new and innovative programs in the tradition of excellence that has characterized the institution throughout its history.

Research at the MBL

The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution

One of the major goals of the Campaign—to create a research center in molecular evolution and comparative molecular biology—has already been accomplished, thanks to a farsighted, \$2 million gift from the Josephine Bay Paul and C. Michael Paul Foundation.

This landmark gift is one of the largest grants ever to be awarded to the MBL. It is catalyzing the

transformation of our existing program in molecular evolution into a larger-scale Center. This new Center is directed by Mitchell Sogin and at present includes the laboratories of Norman Wainwright, Monica Riley, and Neal Cornell.

During the past year, Sogin and his colleagues published a number of papers, including one that describes the phylogenetic affinity of medically important parasites and another that describes the potential convergent evolution of coding regions for an important protein, actin, found in the cytoskeleton of eukaryotes. This exciting research, long supported by the Vetlesen Foundation, was recently again recognized by the National Institutes of Health when it extended Sogin's funding for another 4 years.

The Ecosystems Center

Another goal of the Campaign is to provide support for the Ecosystems Center, the largest resident research unit at the MBL. Its scientists conduct research throughout the world on temperate forests, tropical pastures, estuaries, and arctic tundra. Their goal is understanding—and being able to predict—how ecosystems respond to change, whether it be caused by natural forces or human activity.

Funds raised through the Campaign for the MBL will assure that the Center's outstanding research efforts continue into the 21st Century, a time in which we can expect increasing environmental threats. Funds raised in the Campaign will be used to build new research laboratories, a staging area for field research, a computer classroom, meeting rooms, and geographic information system facilities.

Research progress has moved forward rapidly this past year at the Ecosystems Center, with, for example, John Hobbie and Michele Bahr, in collaboration with Mitch Sogin, identifying previously unidentifiable microbes from Toolik Lake, Alaska, by analyzing their DNA. This cutting-edge technique will help ecologists determine the bacteria responsible for natural processes such as decomposition and enable a better prediction of the effects of changing environmental conditions on



ecosystems processes mediated by bacteria. Edward Rastetter and Matthew Williams have been using computer models to model photosynthetic and other processes in plant canopies. These models are good predictors of natural processes and have enabled a better understanding of the effects of water, clouds, wind, and seasonal change on the productivity of forest stands.

Using a stable isotope of nitrogen in both aquatic and terrestrial ecosystems has enabled scientists Bruce Peterson and Knute Nadelhoffer to trace the movement of nitrogen nutrients. These results have helped to predict the impact of additional inputs of nitrogen on plant growth and species distribution from sources such as acid deposition.

Marine Resources Center

Success in the Campaign for the MBL will enable scientists at the Marine Resources Center to enhance existing research efforts and to increase the pace at which they develop new research models.

A major accomplishment in 1996 was the successful culture of the Hawaiian squid, *Euprymna scolopes*, through its entire life cycle with good survival rates and fertile eggs for the next generation. This breakthrough will enable new questions in developmental biology to be addressed by providing organisms from a well-controlled and stable source. Improvements in the growth of the cuttlefish *Sepia officinalis* and in the maintenance of the toadfish promise to assist scientists who require healthy research models.

This past year, MRC director Roger Hanlon established his own research laboratory which focuses on the biological bases of behavior using cephalopods as

a model system. In addition to studies on the feeding behavior of the Hawaiian squid, which led to the culture breakthrough, he and his collaborators have made progress on four projects: sexual selection and sperm competition among squids, sensory biology of chemoreception in *Nautilus*, observational learning in cuttlefish, and spatial learning in cuttlefish and octopus.

BioCurrents Facility

During 1996, the National Vibrating Probe Facility, directed by Peter J.S. Smith, was renamed the BioCurrents Research Center as part of the renewal of funding by the National Institutes of Health as a NCRR Biomedical Technology Research Program. Funding under this grant renewal will allow Smith and his staff to expand the technical capabilities of the facility and to appoint two new staff members.

Several instruments have been developed or refined in the BioCurrents Research Center during the past year. The ion probe instrumentation was redesigned, new polarographic techniques were developed, and a four-head BioKelvin assembly was created. Smith and colleagues put the equipment to good use, demonstrating the importance of the acidic environment in which sperm develop for proper maturation. These results, obtained in collaboration with Sylvie Breton and her colleagues at Massachusetts General Hospital, present the possibility of developing a male contraceptive protocol in which altering the acidity of the environment in the vas deferens may prevent normal development and maturation of the sperm.

Other developments

Shinya Inoue and Rudolf Oldenbourg of the Architectural Dynamics of Living Cells Program



continued to explore the activities of living cells with the most sophisticated techniques of light microscopy. Highlights, in collaboration with a number of investigators, included observing microtubule and actin bundle dynamics in dividing newt lung epithelial cells and in growth cones of *Aplysia* bag cell neurons. The new polarized light microscope (Pol-Scope) developed by Rudolf Oldenbourg is now available commercially and has been crucial to the group's ability to obtain its exciting new results.

Long-time BioCurrents Research Center collaborator David Keefe became the MBL's newest resident scientist in December of 1996. Keefe is Director of the Laboratory for Reproductive Medicine, Brown University and Women and Infants' Hospital and investigates the mechanisms that may cause age-related female infertility. Using his advanced equipment and techniques with those in the laboratories of Peter Smith and Rudolf Oldenbourg, Keefe is searching for ways to determine the viability of egg cells. His ultimate goal is to enable women to make more informed choices about the reproductive therapies available to them.

Ferenc Harosi and his colleagues expanded upon earlier studies on how fish detect polarized light. During the past year they demonstrated that light reflection upon a dielectric partition may be the optical basis for polarized light detection. Harosi has also been working with Jeffrey Van Keuren on the development of vision in codfish.

The Calcium Imaging Laboratory has focused upon pattern formation of early brains and hearts in the zebrafish and the dorsoventral axis in *Drosophila*. On the theoretical front, Lionel Jaffe has recently recognized a new class of calcium waves which he calls ultraslow waves. Observations of such waves include those waves which help organize the nervous system in *Xenopus* and the eye in *Drosophila*.

Summer research

The MBL remains deeply committed to the summer programs that have nurtured the nation's life sciences throughout the 20th Century. The annual influx of summer researchers is what makes the MBL a great scientific meeting place and center of discovery. The MBL's campaign will strengthen summer research by building an endowment to provide fellowships for the brightest biologists. Funds raised will also build new bridges between summer and year-round research programs.

During the summer of 1996, hundreds of distinguished researchers came to Woods Hole with their graduate students and technicians, their



equipment, their ideas and their passion to learn from each other and from the models the sea provides.

Among those visiting scientists were Lawrence Rome of the University of Pennsylvania and his colleagues at Northern Arizona University. They revealed the first physiological evidence of the extreme adaptations involved in operating the fastest of fast muscle—the toadfish swimbladder muscle. Contrary to popular belief, the swimbladder does little to regulate buoyancy in the toadfish. Rather, the muscles that envelop the swimbladder contract and relax at a remarkable 200 times per second, creating the animal's distinctive “boatwhistle” mating call. These muscles operate almost 100 times faster than the fish's locomotory muscles. To learn more about the physiology of sonic muscles, Rome and his colleagues examined two of the fastest known vertebrate muscles, the toadfish swimbladder, and the shaker muscle of the rattlesnake tail, which rattles at approximately 90 beats per second. They found that the sound-producing muscles of both animals have evolved similar, and extreme, physiological modifications in order to operate at such unusually high frequencies. In superfast sonic muscles, calcium (the intracellular trigger for contraction) is cycled through the system 50 times faster and the molecular crossbridges that generate force between the

muscle filaments cycle about 100 times faster than in locomotory muscles.

While some MBL scientists are exploring the toadfish's noise-making muscles, others are working on the problem of how the grunting fish hears—and what it hears. Summer investigator Richard Fay (Loyola University of Chicago) and his colleagues are interested in the broad question of how vertebrates hear. Fay explores the organs of hearing and the neural wiring that carries information about sound to the brain. Fay and colleagues recently worked out some of the mechanisms by which the toadfish determines the direction from which a sound is coming. They learned that toadfish ears respond to the motion of particles rather than to sound pressure and are able to respond to movements of fine hair cells of one-tenth of one billionth of a meter, similar to the limits of the mammalian ear.

Research fellowships

Twenty scientists came to the MBL in 1996 to do research as participants in the MBL's Research Fellowships Program. These Fellows are funded by income from the endowment and a variety of gifts. Among these is the generous one from the MBL Associates who raise the funds through sales in the gift shop. Fellows work independently on their own research projects and participate in a weekly seminar series where they learn about each other's areas of scientific interests. In 1996 Fellows studied carbon cycling, ion exchange across cellular membranes, motor control systems in shore crabs, muscle physiology, spatial learning in cephalopods, the cell cycle, firing patterns in neurons, development and regeneration in ctenophores, olfactory receptors in lobsters, GABA receptors in skate retinas, and the evolution of crawling sperm in nematodes.

Education at the MBL

Summer courses

Since its founding, the MBL has trained in its courses many of the world's leading biologists, 11 of whom have been awarded Nobel Prizes. The cornerstone of the MBL's current educational program is its six state-of-the-art courses in physiology, neurobiology, parasitology, embryology, neural systems and behavior, and microbiology. Shorter courses that provide students with the latest information and techniques in such diverse fields as medical informatics, computational neuroscience, molecular evolution, microinjection, molecular mycology, and optical microscopy and imaging are also offered.



Every year the MBL assembles the best and brightest scientists from throughout the world to serve as faculty for its advanced, intensive laboratory courses. Because of this extraordinary gathering of expertise, unequalled by any single university, and because of the state-of-the-art equipment provided by major equipment companies, MBL courses attract extremely talented graduate students, postdoctoral fellows, and M.D./Ph.D. candidates. These students, who come from more than 200 major medical schools, universities, and research institutions, are chosen for their significant academic accomplishments and the likelihood of high potential for future scientific productivity.

In 1996, the Burroughs Wellcome Fund awarded a grant of \$1 million in support of the MBL's education program. A large portion of the grant is being used as endowment. These funds will allow the Laboratory to continue its tradition of training excellent scientists—individuals who bring new ideas and energy to the field and who are just beginning to extend or challenge familiar lines of inquiry in the biomedical sciences.

During the campaign, additional gifts will be sought to increase the endowment to provide long-term stability for the educational program.

The generous funding by the Burroughs Wellcome fund was supplemented by its support for the Biology of Parasitism course and the new course in Molecular Mycology. Other generous funders of the courses include the Howard Hughes Medical Institute, the Grass Foundation, the William Randolph Hearst Foundation, the National Institutes of Health, NASA, the U.S. Department of Energy, the Office of Naval Research, the National Library of Medicine, and the National Science Foundation.

Semester in Environmental Science

For more than 20 years, the MBL has been host to top-notch undergraduates in the Boston University Marine Program. The MBL is now also venturing into undergraduate education, through the Ecosystems Center. Beginning in the fall of 1997, undergraduates from a number of liberal arts colleges will spend a semester studying environmental sciences with Center researchers. With an increase in interest in the environment, most schools have found they don't have enough faculty to offer the in-depth ecological instruction that their students need. The MBL is prepared to fill the void. The goal of the Semester in Environmental Sciences program is to attract bright young people to the field of ecosystems science and to equip all participants—whether they choose to become career scientists or not—with the skills to act as enlightened citizens when considering or addressing environmental issues. This program has initially been made possible by grants amounting to more than \$1 million from the Andrew W. Mellon Foundation.

MBL/WHOI Library

The Library is one of the finest scientific libraries in the world, housing an exceptionally valuable collection



in biology, especially in journals. The challenge is to maintain the traditional print library, while energetically expanding and exploiting the electronic library. Towards that end, a grant from the National Library of Medicine made it possible to develop the Marine Animal Web Page, a knowledge database of more than 200 marine animals that researchers and students can access over the Internet to obtain images, genomic information, current literature citations, and ordering information through the MBL. This advanced product realizes the potential for the Library to go beyond archival collections and, instead, develop custom information for researchers and students working in laboratories throughout the world.

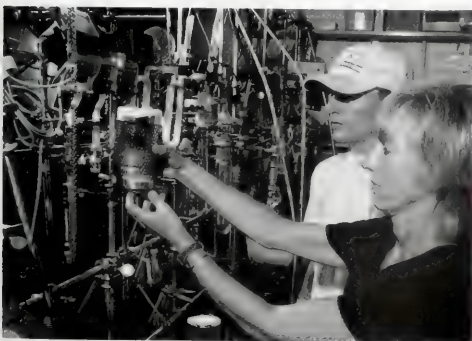
Facilities

The updating and maintenance of our facilities continues to be an important objective of the Laboratory. The Trustees have recognized the need to improve our existing facilities and to undertake a limited amount of new construction and have incorporated these as important goals for the Campaign.

Efforts continued in 1996 to help correct some of the deferred maintenance problems on campus, as well as the upgrading of our facilities. The results are apparent in a remodeled Veeder House, an air-conditioned Whitman Auditorium, the energy-efficient electric lights retrofitted in Swope, Lillie, and Loeb, and the new windows in Loeb. These windows are part of a larger effort to provide a new heating, ventilation, and air conditioning system in the Loeb Laboratory, a project being partially funded by the National Science Foundation and Colonial Gas.

MBL Trustees

The Board of Trustees welcomed three new members to the Board in 1996. Former Chairman of the Federal



Reserve Board and Secretary of the U.S. Treasury, Mr. G. William Miller, was appointed to serve as a member of the Class of 2000. MBL Corporation Member Dr. Porter W. Anderson, Jr., was elected to serve as a member of the Class of 2001. Dr. Gerald Weissmann was also reappointed to the Board in 1996 and will serve as a member of the Class of 2001. Ms. Mary B. Conrad, Senior Vice President of Fiduciary Trust International, was appointed Treasurer of the Corporation. Ms. Conrad follows the tenure of Robert Manz who retired after serving as an outstanding and committed Treasurer for nearly 10 years.

Special thanks are due to Irving Rabb and Tom Pollard whose terms on the Board ended. Mr. Rabb served as a board member for ten years, bringing his wisdom, business acumen, and understanding of non-profit organizations to all discussions, while Dr. Pollard served for seven years and was key in the scientific planning for the future of the Laboratory.

Staff

The MBL welcomed Timothy J. Roddy to the staff in 1996. Tim is MBL's first Chief Financial Officer. He comes to us from the National Academy of Sciences where he served since 1989, most recently as Director of

the Accounting Office. Tim replaces MBL Controller John Speer, who retired in June of 1996 after 14 years of distinguished service at the Laboratory.

Looking Ahead

For the past two years, following our historic change in governance, the MBL's Board of Trustees, Science Council, and Long-Range Planning Committee, have been focusing on and planning for the future of the Laboratory. Together we are committed to systematic growth and change while preserving the special roles that the Laboratory has played in its 109-year history.

The upcoming Campaign for the Marine Biological Laboratory is crucial to achieving those goals. It will require the strength and commitment of a dedicated group of individuals who believe in the MBL and the special contribution the Laboratory can make to the benefit of humanity. For the first time, the Marine Biological Laboratory will be asking scientists and friends of science to make a major investment in the future of the Laboratory. I look forward to working with you as we move into the 21st Century.

—John E. Burris



Report of the Treasurer

The Treasurer's report accompanies the financial statements for the Marine Biological Laboratory for the year ended December 31, 1996.

As reported in 1995, the Laboratory has adopted the reporting format mandated by the Financial Accounting Standards Board, the purpose of which is to standardize reporting of not-for-profit institutions. Activities are categorized as to how the net assets of the institution are affected. A review of these three subdivision types follows.

(1) *Unrestricted* net assets are immediately at the MBL's disposal; the funds for normal operating activities and balances are included hereunder.

(2) *Temporarily restricted* net assets are those that are accompanied by some granting or donating institution's limitation on the manner or the time period in which the institution may use such funds; the unexpected balance of a multi-year grant for a specific program of research would be an example of this category.

(3) *Permanently restricted* net assets represent funds that may not be spent by the MBL, although the income deriving from investment of these funds may be spent. The original corpus of permanent endowment funds is an example of this category.

Having adopted additional reporting requirements in 1995, pledges are booked by the institution as an asset, even though the cash has yet to be received, similar to an account receivable.

The status of these net assets is presented in the Balance Sheet; activities which affected them are presented in the Statement of Activities and the manner in which these activities changed the cash position of the Laboratory is presented in the Statement of Cash Flows.

In 1996 the MBL had another good year. The net assets of the Marine Biological Laboratory increased 13% in 1996 from \$48.2 million to \$54.5 million. This continuing success is due to the strength of the stock market, combined with aggressive investment

management activities. Our long-term investments increased \$4.4 to \$29.8 million and had a return of 17.5%. Although this was not up to the stellar performance of 1995, when we enjoyed a return of 30.2%, our investment managers beat their benchmarks. As of December 31, 1996, the investment portfolio is invested 67% in equities, 30% fixed income, and 3% cash. The continued success is also due to gifts and pledges resulting from the early stages of our Capital Campaign, which increased current assets 36% or \$2.2 million.

Depreciation of \$1.4 million caused *unrestricted* net assets to decrease by \$938,298 in 1996 to \$19.4 million. In accordance with Financial Accounting Standards, the Laboratory is reporting the results of operations after depreciation. While this highlights the need to generate funds for the replacement of our scientific and other facilities, it masks a strong and healthy result of operations: a surplus of \$.2 million for the year. We will be investing this surplus in several key capital projects during 1997, the fourth year we have been able to make infusions into such programs. Other operational achievements included the successful implementation of a new financial information system, the receipt of a \$2 million gift from the Paul Bay Foundation for the Center in Molecular Evolution, as well as several gifts in support of the groundbreaking Semester in Environmental Science.

Since the last Treasurer's report, several key personnel changes have occurred. John Speer retired after 14 years of dedicated service and Timothy Roddy joined the MBL in the newly created position of Chief Financial Officer. And after nine rewarding years, Robert Manz yielded the Treasurer's hat to me.

I am looking forward to my role as the Treasurer and believe the MBL is in a sound position to move aggressively forward by generating further capital to be used in support of its longstanding mission of superb biological research and education.

—Mary B. Conrad

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REPORT OF INDEPENDENT ACCOUNTANTS

To the Board of Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have audited the accompanying balance sheet of Marine Biological Laboratory (the "Laboratory") as of December 31, 1996 and the related statements of activities and cash flows for the year then ended. We previously audited and reported upon the financial statements of the Laboratory for the year ended December 31, 1995, which condensed statements are presented for comparative purposes only. These financial statements are the responsibility of the Laboratory's management. Our responsibility is to express an opinion on these financial statements based on our audit.

We conducted our audit in accordance with generally accepted auditing standards. Those standards require that we plan and perform the audit to obtain reasonable assurance about whether the financial statements are free of material misstatement. An audit includes examining, on a test basis, evidence supporting the amounts and disclosures in the financial statements. An audit also includes assessing the accounting principles used and significant estimates made by management, as well as evaluating the overall financial statement presentation. We believe that our audit provides a reasonable basis for our opinion.

In our opinion, the financial statements referred to above present fairly, in all material respects, the financial position of Marine Biological Laboratory at December 31, 1996, and its activities and cash flows for the year then ended in conformity with generally accepted accounting principles.

Our audit was conducted for the purpose of forming an opinion on the basic financial statements taken as a whole. The supplemental schedules for unrestricted net assets (Schedule I), temporarily restricted net assets (Schedule II), and permanently restricted net assets (Schedule III) as of December 31, 1996 are presented for purposes of additional analysis and are not a required part of the basic financial statements. Such information has been subjected to the auditing procedures applied in the audit of the basic financial statements and, in our opinion, is fairly stated, in all material respects, in relation to the basic financial statements taken as a whole.

Boston, Massachusetts
March 28, 1997

Coopers & Lybrand L.L.P.

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MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1996

(with comparative totals for 1995)

ASSETS	1996	1995
Cash and cash equivalents	\$ 391,528	\$ 844,528
Short-term investments, at market (Note C)	3,918,194	1,552,459
Accounts receivable, net of allowance for doubtful accounts of \$15,000 in 1996 and \$10,000 in 1995	762,860	742,371
Pledges receivable (Note H)	1,826,494	1,049,127
Receivables due for costs incurred on grants and contracts	1,114,082	1,616,678
Other assets	482,992	467,052
Total current assets	8,496,150	6,272,215
Long-term investments, at market (Notes C and D)	29,763,495	25,352,429
Pledges receivable (Note H)	2,406,350	1,457,147
Plant assets (Note F):		
Land	702,908	702,908
Buildings	31,730,830	31,730,830
Equipment	4,270,278	3,496,593
	36,704,016	35,930,331
Less accumulated depreciation	(16,008,392)	(14,534,514)
Plant assets, net	20,695,624	21,395,817
Total long-term assets	52,865,469	48,205,393
Total assets	\$61,361,619	\$54,477,608
LIABILITIES AND NET ASSETS	1996	1995
Current portion of long-term debt (Note F)	\$ 173,611	\$ 76,670
Current obligations held under capital leases (Note E)	30,497	33,101
Accounts payable and accrued expenses	1,536,015	1,945,977
Deferred income	179,606	239,145
Advances on contracts	142,392	195,467
Total current liabilities	2,062,121	2,490,360
Annuities and unitrusts payable	959,513	1,076,320
Long-term debt (Note F)	2,565,210	2,320,000
Obligations held under capital leases (Note E)	26,763	29,470
Advances on contracts	1,210,950	400,000
Total long-term liabilities	4,762,436	3,825,790
Total liabilities	6,824,557	6,316,150
Commitments and contingencies (Note E)		
Net assets:		
Unrestricted	19,371,978	20,310,276
Temporarily restricted	21,484,748	15,882,483
Permanently restricted	13,680,336	11,968,699
Total net assets (Note I)	54,537,062	48,161,458
Total liabilities and net assets	\$61,361,619	\$54,477,608

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF ACTIVITIES

for the year ended December 31, 1996

(with comparative totals for the year ended December 31, 1995)

	<u>Unrestricted</u>	<u>Temporarily Restricted</u>	<u>Permanently Restricted</u>	<u>1996 Total</u>	<u>1995 Total</u>
Operating support and revenues:					
Government grants	\$ 9,250,585			\$ 9,250,585	\$ 8,194,978
Private contracts	1,008,173			1,008,173	1,007,556
Laboratory rental income	1,489,069			1,489,069	1,520,880
Tuition	220,044			220,044	107,725
Fees for services	3,779,934			3,779,934	3,483,165
Gifts	574,053	\$ 2,603,635	\$ 954,739	4,132,427	1,324,004
Pledges		2,839,302		2,839,302	2,466,782
Investment income	149,045	224,324		373,369	382,841
Miscellaneous revenue	346,124			346,124	373,336
Total operating support and revenues	16,817,027	5,667,261	954,739	23,439,027	18,861,267
Total return income and gains used for operations	38,992	1,042,804		1,081,796	727,066
Net assets released from restrictions	3,183,976	(3,183,976)			
Present value adjustment to annuities		118,076		118,076	(257,548)
	<u>20,039,995</u>	<u>3,644,165</u>	<u>954,739</u>	<u>24,638,899</u>	<u>19,330,785</u>
Expenses:					
Research	9,442,484			9,442,484	8,818,418
Instruction	2,609,733			2,609,733	2,789,263
Services	4,570,423			4,570,423	4,231,938
Administration	4,141,544			4,141,544	3,600,152
Other	279,524			279,524	130,963
Total expenses	<u>21,043,708</u>			<u>21,043,708</u>	<u>19,570,734</u>
Excess (deficit) of support and revenue over expenses before gain on investments	(1,003,713)	3,644,165	954,739	3,595,191	(239,949)
Nonoperating:					
Investment income	38,992	1,125,155		1,164,147	375,844
Net realized and unrealized gains (losses) on investments	65,415	1,875,749	756,898	2,698,062	4,937,094
Total return income and gains used for operations	<u>(38,992)</u>	<u>(1,042,804)</u>		<u>(1,081,796)</u>	<u>(727,066)</u>
Reinvested income and gains	65,415	1,958,100	756,898	2,780,413	4,585,872
Change in net assets	(932,298)	5,602,265	1,711,637	6,375,604	4,345,923
Cumulative adjustment for pledges				—	1,310,492
Net assets, beginning of year	<u>20,310,276</u>	<u>15,882,483</u>	<u>11,968,699</u>	<u>48,161,458</u>	<u>42,505,043</u>
Net assets, end of year	<u>\$19,371,978</u>	<u>\$21,484,748</u>	<u>\$13,680,336</u>	<u>\$54,537,062</u>	<u>\$48,161,458</u>

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CASH FLOWS

for the year ended December 31, 1996

(with comparative totals for 1995)

	1996	1995
Cash flows from operating activities:		
Change in net assets	\$ 6,375,604	\$ 4,345,923
Adjustments to reconcile change in net assets to net cash (used by) from operating activities:		
Depreciation	1,473,878	1,410,049
Unrealized (gain) loss on investments	(1,765,532)	(3,610,830)
Realized (gain) loss on investments	(932,530)	(1,326,264)
Present value adjustment to annuities payable	(118,076)	257,548
Contributions restricted for long-term investment and annuities	(1,042,945)	(73,884)
Provision for bad debt	5,000	—
Change in certain balance sheet accounts:		
Accounts receivable	(25,489)	66,845
Pledges receivable	(1,726,570)	(2,506,274)
Grants and contracts receivable	502,596	(602,355)
Other assets	(15,490)	(12,045)
Accounts payable and accrued expenses	(409,962)	134,940
Deferred income	(59,539)	(7,601)
Advances on contracts	757,875	574,357
Net cash provided by (used in) operating activities	3,018,370	(1,349,591)
Cash flows from investing activities:		
Purchase of property and equipment	(730,966)	(709,122)
Disposals of property and equipment	—	4,500
Proceeds from sale of investments	7,293,473	42,882,597
Purchase of investments	(11,347,575)	(40,515,256)
Net cash provided by (used in) investing activities	(4,785,068)	\$ 1,662,719
Cash flows from financing activities:		
Payments on annuities and unitrusts payable	(23,369)	(23,609)
Receipt of permanently restricted gifts	954,739	51,415
Annuity and unitrusts donations received	88,206	22,469
Loan proceeds	500,000	—
Payments on long-term debt	(157,849)	(79,011)
Payments on capital leases	(48,029)	(29,066)
Net cash provided by (used in) financing activities	1,313,698	(57,802)
Net (decrease) increase in cash and cash equivalents	(453,000)	255,326
Cash and cash equivalents at beginning of year	844,528	589,202
Cash and cash equivalents at end of year	\$ 391,528	\$ 844,528

The accompanying notes are an integral part of the financial statements.

Marine Biological Laboratory

Notes to Financial Statements

A. Purpose of the Laboratory

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

B. Significant Accounting Policies

Basis of Presentation

The accompanying financial statements have been prepared on the accrual basis and in accordance with the principles of not-for-profit accounting.

Net Assets

The Laboratory's net assets are segregated into three groups:

Unrestricted

Unrestricted net assets represent the results of operations, assets released from restrictions, and all resources not subject to donor-imposed restrictions of a more specific nature than the furtherance of the Laboratory's mission. Included in unrestricted net assets are the funds related to the \$20,695,624 of net plant assets, offset by debt and other liabilities.

Temporarily Restricted

These assets include gifts plus monies for which the specific, donor-imposed restrictions have not been met and pledges, annuities, and unitrusts for which the ultimate purpose of the proceeds is not permanently restricted. As the restrictions are met, the assets are released to unrestricted net assets. Also, realized/unrealized gains/losses associated with permanently restricted gifts which are not required to be added to principal by the donor are classified as temporarily restricted but maintain the donor requirements for expenditure.

Permanently Restricted

These assets include gifts, pledges and trusts which require that the corpus be invested in perpetuity and only the income be made available for program operations in accordance with donor restrictions.

Nonoperating revenues include realized and unrealized gains on investments during the year as well as investment income on the master pooled investments. Investment income from short-term investments and investments held in trust by others is included in operating support and revenues. To the extent that nonoperating investment income and gains are used for operations as determined by the Laboratory's total return utilization policy (see below), they are reclassified from nonoperating to operating on the statement of activities as "Total return income and gains used for operations." All other activity is classified as operating revenue. The Laboratory recorded net realized gains of \$932,530 and \$1,326,264 and net unrealized gains of \$1,765,532 and \$3,610,830 in 1996 and 1995, respectively.

Cash and Cash Equivalents

Cash equivalents consist of resources invested in overnight repurchase agreements and other highly liquid investments with original maturities of three months or less.

Financial instruments which potentially subject the Laboratory to concentrations of risk consist primarily of cash and investments. The Laboratory maintains cash accounts with one banking institution. Investments are maintained primarily with two institutions.

Investments

In 1996, the Laboratory adopted SFAS 124, "Accounting for Certain Investments Held by Not-for-Profit Organizations." The impact of adoption was immaterial to the financial position and changes in net assets.

Investments purchased by the Laboratory are carried at market value. Donated investments are recorded at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the first-in, first-out method. Investments with an original maturity of three months to one year are classified as short-term. All other investments are considered long-term.

In 1924, the Laboratory became the beneficiary of certain investments, classified as permanently restricted net assets, which are held in trust by others. The Laboratory has the continuing rights to the income produced by these funds in perpetuity, subject to the contractual restrictions on the use of such funds. Accordingly, the trust has established a process to conduct a review every ten years by an independent committee to ensure the Laboratory continues to perform valuable services in biological research in accordance with the restrictions placed on the funds by the agreement. The committee met in 1994 and determined that MBL has continued to meet the contractual requirements. The market values of such investments are \$6,214,477 and \$5,457,579 at December 31, 1996 and 1995, respectively. The income on these investments totaled \$224,324 and \$186,505 in 1996 and 1995, respectively.

Investment Income and Distribution

For the master pooled investments, the Laboratory employs a total return utilization policy that establishes the amount of the investment return made available for spending each year. The Finance and Investment Committee has approved a standing policy that the withdrawal will be based on 4% to 5% of the latest three-year average ending market values of the funds. The market value includes the principal plus reinvested income, realized and unrealized gains and losses. Spending rates in excess of 5%, but not exceeding 7%, can be utilized if approved in advance by the Finance and Investment Committee of the Trustees. For fiscal 1996, the Laboratory obtained approval to expend 6% of the latest three-year average ending market values of the investments. The 6% includes a 1½% administration fee for endowments. This fee was approved by the Laboratory's Board of Trustees for the years 1996–2000.

The net appreciation on permanently and temporarily restricted net assets is reported together with temporarily restricted net assets until such time as all or a portion of the appreciation is distributed for spending in accordance with the total return utilization policy and applicable state law.

Investment income on the pooled investment account is allocated to the participating funds on the market value unit basis (Note D).

Plant Assets

Buildings and equipment are recorded at cost. Donated facility assets are recorded at fair market value at the date of the gift. Depreciation is computed using the straight-line method, beginning the month after the asset is placed in service, over the asset's estimated useful life. Estimated useful lives are generally three to ten years for equipment and 20 to 40 years for buildings and improvements. Depreciation expense for the year ended December 31, 1996 amounted to \$1,473,878 and has been recorded in the statement of activities in the appropriate functionalized categories. When assets are sold or retired, the cost and accumulated depreciation are removed from the accounts and any resulting gain or loss is included in unrestricted income for the period.

Annuities and Unitrusts Payable

Amounts due to donors in connection with gift annuities and unitrusts are determined based on remainder value calculations, as of December 31, 1996, with varied assumptions of rates of return and payout terms.

Advances on Contracts

Advances on contracts includes funding received for grants and contracts before it is earned. In certain circumstances, long-term advances are invested in the master pooled account until they are expended.

Revenue Recognition

Revenue is recognized at the time it is earned. The sources of revenue include grant payments from governmental agencies, contracts from private organizations, and income from the rental of laboratories and classrooms for research and educational programs. The tuition income is net of student financial aid of \$479,000 and \$499,000 in 1996 and 1995, respectively. Fees are received from the following activities: housing, dining, library, scientific journals, aquatic resources and research services.

Use of Estimates

The preparation of financial statements in conformity with generally accepted accounting principles requires management to make estimates and assumptions that affect the reported amounts of assets and liabilities and disclosure of contingent assets and liabilities at the date of financial statements and the reported amounts of revenues and expenses during the reporting period. Actual results could differ from those estimates.

Tax-Exempt Status

The Laboratory is exempt from federal income tax under Section 501(c)(3) of the Internal Revenue Code.

C. Investments

The following is a summary of the cost and market value of investments at December 31, 1996 and 1995:

	<i>Market</i>		<i>Cost</i>	
	<i>1996</i>	<i>1995</i>	<i>1996</i>	<i>1995</i>
Certificates of deposit	\$ 55,350	\$ 52,459	\$ 55,350	\$ 52,459
Money market securities	3,469,588	1,530,048	3,469,589	1,530,048
U.S. Government securities	1,233,690	1,206,863	998,829	998,829
Corporate fixed income	3,431,333	3,342,219	3,278,396	3,197,937
Common stocks	3,793,156	2,770,153	2,742,841	2,202,312
Mutual funds	17,371,972	14,305,946	14,555,958	12,183,166
Limited partnerships	<u>\$4,326,600</u>	<u>\$3,697,200</u>	<u>\$3,108,464</u>	<u>\$3,028,464</u>
Total investments	<u>\$33,681,689</u>	<u>\$26,904,888</u>	<u>\$28,209,427</u>	<u>\$23,193,215</u>

Investment portfolios for the years ended December 31, 1996 and 1995 are as follows:

Current Investments

Certificates of deposit	\$ 55,350	\$ 52,459	\$ 55,350	\$ 52,459
Money market 1784 Fund	2,755,593	500,000	2,755,593	500,000
Common stocks	50,608	—	50,608	—
STAR mutual fund	<u>1,056,643</u>	<u>1,000,000</u>	<u>1,063,089</u>	<u>1,004,309</u>
Total	<u>\$ 3,918,194</u>	<u>\$ 1,552,459</u>	<u>\$ 3,924,540</u>	<u>\$ 1,556,768</u>

	Market		Cost	
	1996	1995	1996	1995
<i>Long-Term Investments</i>				
Pooled investments:				
Master pooled investments	\$21,858,658	\$18,231,994	\$17,889,881	\$15,493,704
Separately invested:				
General Chase trust	4,898,630	4,311,663	3,926,959	3,736,796
Library Chase trust	1,315,847	1,145,916	1,065,008	1,004,351
Annuity investments	1,690,360	1,662,856	1,402,939	1,401,596
Total	29,763,495	25,352,429	24,284,787	21,636,447
Total investments	<u>\$33,681,689</u>	<u>\$26,904,888</u>	<u>\$28,209,427</u>	<u>\$23,193,215</u>

D. *Accounting for Pooled Investments*

Certain net assets are pooled for investment purposes. Investment income from the pooled investment account is allocated on the market value unit basis, and each fund subscribes to or disposes of units on the basis of the market value per unit at the beginning of the calendar quarter within which the transaction takes place. The unit participation of the funds at December 31, 1996 and 1995 is as follows:

	1996	1995
Unrestricted	4,415	4,495
Temporarily restricted	41,426	43,242
Permanently restricted, restricted income	66,165	63,766
Permanently restricted, unrestricted income	277	229
Advances on contracts	6,493	—
	<u>118,776</u>	<u>111,732</u>

Pooled investment activity on a per-unit basis was as follows:

	1996	1995
Unit value at beginning of year	\$ 159.37	\$ 127.43
Unit value at end of year	<u>184.03</u>	<u>159.37</u>
Increase in realized and unrealized appreciation	24.66	31.94
Income earned on pooled investments	1.87	0.11
Total return on pooled investments	<u>\$ 26.53</u>	<u>\$ 32.05</u>

E. *Commitments and Contingencies**Capital Leases*

As of December 31, 1996 the Laboratory had capital leases for office equipment. Interest rates on the obligations are between 1.55% and 6.63%. The future minimum lease payments as of December 31, 1996 are as follows:

1997	\$30,497
1998	18,427
1999	7,211
2000	1,125
	<u>\$57,260</u>

F. *Long-Term Debt*

Long-term debt consisted of the following at December 31:

	1996	1995
Variable rate (5.36% at December 31, 1996) Massachusetts Industrial Finance Authority Series 1992A Bonds payable in annual maturities through 2012	\$ 990,000	\$1,020,000
6.63% Massachusetts Industrial Finance Authority Series 1992B Bonds, payable in annual maturities through 2012	1,330,000	1,375,000
5.8% The University Financing Foundation, Inc., payable in monthly installments through 2000	418,821	—
Other note payable	—	1,670
	<u>\$2,738,821</u>	<u>\$2,396,670</u>

The aggregate amount of principal due on long-term debt (not including capital leases) for each of the next five fiscal years and thereafter is as follows:

1997	\$ 173,611
1998	184,097
1999	195,095
2000	216,355
2001	119,663
Thereafter	<u>1,850,000</u>
	2,738,821
Less current portion	<u>(173,611)</u>
Total	<u>\$2,565,210</u>

In 1992, the Laboratory issued \$1,100,000 Massachusetts Industrial Finance Authority (MIFA) Series 1992A Bonds and \$1,500,000 MIFA Series 1992B. These bonds pay varying annual interest rates ranging from 3.48% to 6.63%. Interest expense on this debt totaled \$148,007 for the year ended December 31, 1996. The Series 1992 A and B Bonds mature on December 1, 2012 and are collateralized by a first mortgage on certain Laboratory property.

The agreements related to these Bonds subject the Laboratory to certain covenants and restrictions. Under the most restrictive covenant of this debt, the Laboratory's operating surplus (before transfers), interest, expense and transfers from the quasi-endowment for debt service must equal or exceed all debt service payments, as defined by the agreement. The Laboratory was in compliance with these covenants and restrictions at December 31, 1996.

In 1996, the Laboratory borrowed \$500,000 with an interest rate of 5.8% from the University Financing Foundation, Inc. The interest expense for the year ended December 31, 1996 was \$24,640. The Laboratory has pledged 50,000 shares of a fixed income fund with a market value in excess of debt at December 31, 1996 as collateral. Under the most restricted covenant to the agreement, the collateral must be free and clear of all liens. The Laboratory was in compliance with all covenants of this agreement at December 31, 1996.

The Laboratory has a line of credit agreement with the Bank of Boston from which it may draw up to \$1,000,000. No amounts were outstanding under this agreement as of December 31, 1996 and 1995.

G. *Retirement Plan*

The Laboratory participates in the defined contribution pension plan of TIAA-CREF (the "Plan"). The Plan is available to permanent employees who have completed two years of service. Under the Plan, the Laboratory contributes 10% of total compensation for each participant. Contributions amounted to \$661,089 and \$571,285 for the years ended December 31, 1996 and 1995, respectively.

H. *Pledges*

Unconditional promises are included in the financial statements as pledges receivable and revenue of the appropriate net asset category. Pledges are recorded after discounting to the present value of the future cash flows. Unconditional promises are expected to be realized in the following periods:

	<i>1996</i>	<i>1995</i>
In one year or less	\$1,836,874	\$1,054,472
Between one year and five years	<u>2,780,000</u>	<u>1,669,700</u>
	4,616,874	2,724,172
Less: discount of \$373,650 in 1996 and \$212,553 in 1995 and allowance of \$10,380 in 1996 and \$5,345 in 1995	<u>(384,030)</u>	<u>(217,898)</u>
	<u>\$4,232,844</u>	<u>\$2,506,274</u>

Pledges receivable at December 31 have the following restrictions:

Research and education	\$4,232,844	\$2,501,674
Permanently restricted net assets		<u>4,600</u>
	<u>\$4,232,844</u>	<u>\$2,506,274</u>

1. *Postretirement Benefits:*

The Laboratory accounts for its postretirement benefits under Statement No. 106, "Employers' Accounting for Postretirement Benefits Other than Pensions," which requires employers to accrue, during the years that the employee renders the necessary service, the expected cost of benefits to be provided during retirement. As permitted, the Laboratory has elected to amortize the transition obligation over 20 years commencing on January 1, 1994.

The Laboratory's policy is that all current retirees and certain eligible employees who retired prior to June 1, 1994 will continue to receive postretirement health benefits. The remaining current employees will receive benefits; however, those benefits will be limited as defined by the Plan. Employees hired on or after January 1, 1995 will not be eligible to participate in the postretirement medical benefit plan.

Net postretirement benefits for 1996 and 1995 include:

	<u>1996</u>	<u>1995</u>
Service cost (benefits earned during period)	\$ 61,712	\$ 61,851
Interest cost (on projected benefit obligation)	149,149	141,218
Actual return on plan assets	(20,700)	(13,801)
Net amortization and deferral	92,176	84,953
Net postretirement benefits cost	<u>\$ 282,337</u>	<u>\$ 274,221</u>

Below is a reconciliation of the funded status of the Plan at December 31, 1996 and 1995:

Accumulated postretirement benefit obligation:		
Retirees and dependents	\$1,369,787	\$1,435,418
Fully eligible active participants	230,290	225,602
Other active participants	608,796	557,950
Total	2,208,873	2,218,970
Market value of plan assets	588,337	398,785
Assets less than obligations	1,620,536	1,820,185
Unrecognized prior service cost (credit)	—	—
Unrecognized net (gain) loss	136,158	257,322
Unrecognized transition obligation	1,475,981	1,562,803
Accrued postretirement benefit cost	<u>\$ (8,397)</u>	<u>\$ (60)</u>

The health care cost trend rate assumptions used in determining the projected benefit obligation begins at 10.0% in 1996 and gradually decreases to 5.0% in the year 2006 and thereafter. The effect of raising the assumed health care cost trend rate by one percentage point in each year would be to increase the accumulated postretirement benefit obligation as of December 31, 1996 by \$202,545 and to increase the aggregate of the service and interest cost components of net periodic postretirement benefit cost for the year then ended by \$18,635. The discount rate used in determining the accumulated postretirement benefit obligation is 7.5%, and the expected return on plan assets was 8.0%. During 1996, the Laboratory contributed \$274,000 to fund the Trust for these postretirement benefits.

Supplemental schedules I, II, and III are not included in this report.

For a complete set of audited financial statements contact:

*Timothy Roddy
Chief Financial Officer
Marine Biological Laboratory
Woods Hole, MA 02543*



Report of the Library Director

In a short time the Library has moved beyond being a physical location to one that now includes electronic resources accessible far from Woods Hole. Thanks to the success of the Internet, scientists now frequently gain access to library information electronically. Unfortunately, the popularity of the Internet has presented its own challenges. What once was a "direct road" for information exchange between scientists and engineers has developed into a super highway and become a billion dollar industry. The World Wide Web is now being pushed beyond its limits, resulting in delays and access problems. The Internet and its proliferation of information and general search engines have created an atmosphere of relative chaos for the casual information seeker and one of frustration for the researcher with more specific information requirements.

If the electronic library is to be kept alive, the skills of the traditional library workers who catalog, index, and collect will have to be merged with those of the computer scientists who have the ability to automate these indexing and storing tasks while managing network overloads. The merging of these two perspectives will preserve the access to information that is needed in daily research.

The library is now developing its own intranet that allows the delivery of scientific information via the WWW to local databases: licensed full-text on-line journals, abstracting and indexing services, etc. This effort seeks to combine the same kinds of skills as a subject specialist in collection development along with the latest technology in digital imaging storage. The concept of a domain-specific intranet has become necessary since the WWW has developed beyond the "free" distribution method of information. This infrastructure is allowing publishers to develop a framework that allows library users to access copyrighted publications without infringing on contractual arrangements between the library and publisher.

A year ago the library installed a new distributed client server computer system named MARINER. The

system is now fully operational. Scientists can use the system from computers in the library or from computers located anywhere in the world through the WWW (mblwhoilibrary.mbl.edu). The system also provides access to commercial databases like BIOSIS and GEOREF.

Journals

The prices of our journals continue to rise at an unprecedented rate. We see no relief in sight. As a result, our ability to continue to order valuable journals continues to shrink. The total number of active journals either purchased or received as gifts or exchanges is now 1,763. Accepting journals electronically does little to decrease costs, but it is more convenient. Such journals are available to all users of the library through the network. All records of journals that are available over the net are linked via a web connection both in the bibliographic record and included in a separate e-journal database developed by our Information Systems Division and the Serials department.

The Boston Library Consortium initiated a new delivery service this past year with the daily delivery of interlibrary loans to Woods Hole. Our interlibrary loan traffic has increased by one third this year due to consortium-wide subscription cutbacks.

Policies

Journal retention, selection and review policies occupied the attention of the Joint Users Committee during this past year. New policy issues involving the loan of materials to other Cape Cod libraries, the use of materials from the Rare Book Room, circulation, library access, and access to the new Special Collection area in the monograph section of the stacks were discussed and approved. Policies are being developed in the area of records retention and the storage and retrieval of archival records.

The Archives, Preservation, and Rare Books Collection

For the first time the Rare Books collection is being cataloged for on-line retrieval of the bibliographic information. More than two hundred books and journals have been rebound or repaired in this special collection primarily through a donor memorial program.

WHOI Branch Libraries

The addition of a new archivist in the WHOI Archives has brought about some changes in record retention policies and the creation of some new programs. The Library began to publish manuscript collections starting with MC NO#1, which detailed the Allyn C. Vine collection of papers in the library. With the new library system in place we have been able to put the catalog of the historic scientific instrument collection on line. The new image server will allow those records to be linked to the images of these historic instruments.

Instruction

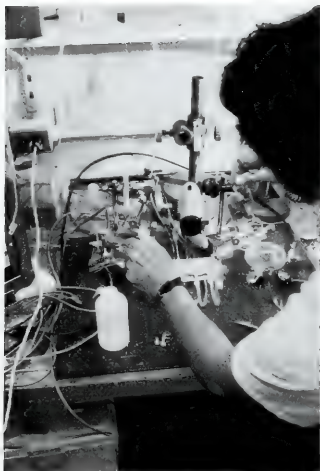
The library staff continues to expand its instructional program. A new Fisheries librarian has been added to the staff to address the information needs of scientists at

the National Marine Fisheries Service in Woods Hole. Because access to the scientific information is changing dramatically and access to information is not as straight-forward as opening the pages of a journal, demonstrations and courses on the new electronic information sites are held monthly. Introductory courses on using the WWW and its home pages are some of the most popular courses offered.

The Future

The electronic revolution now fully underway is proving to be not only a revolution in the library but in the nature of learning and education. Scholars and students are consulting the Internet more often than books. The last time a "technology innovation" like this happened was the invention of the printing press, which lead to the creation of libraries. Just as the printing press made information available to literate people so the Internet is making vast amounts of information available to computer-literate people. The MBL/WHOI Library will continue to play a lead role in collecting and delivering relevant information in an organized way to researchers and students so that the new virtual library complements rather than supplants the virtues of the traditional library.

—Catherine Norton



Educational Programs

Summer Courses

Biology of Parasitism: Modern Approaches (June 13–August 16)

Director

Steven Hajduk, University of Alabama, Birmingham

Course Faculty

Robert Bullis, Marine Biological Laboratory
Harry Dickerson, University of Georgia
Steve Ealick, Cornell University
Patricia Johnson, University of California School of Medicine,
Los Angeles
Keith Joiner, Yale University School of Medicine
Susan Little, University of Georgia
Phil LoVerde, State University of New York, Buffalo
Susan Rohrer, Merck Research Labs
Phillip Scott, University of Pennsylvania
Christian Tschudi, Yale School of Medicine
Buddy Ullman, Oregon Health Sciences University
Elisabetta Ullu, Yale University School of Medicine

Teaching Assistants

Peter Bradley, University of California, Los Angeles
Laura Rosa Brunet, Cornell University
Nino Campobasso, Cornell University
Darrick Carter, Oregon Health Sciences University
Merle Ellosa, University of Pennsylvania
Helen Kwon, Yale School of Medicine
Wendy Leavitt, State University of New York, Buffalo
Katherine Rogers, Yale School of Medicine
Elizabeth Sabin, Cornell University
Anthony Sinai, Yale University School of Medicine
Andrea Smith, University of Alabama, Birmingham
Robin Vora, Cornell University

Lecturers

Abul Abbas, Brigham & Women's Hospital
Norma Andrews, Yale University School of Medicine

Jay Bangs, University of Wisconsin, Madison
Stephen Beverley, Harvard University School of Medicine
John Boothroyd, Stanford University
George Cross, Rockefeller University
Karen Day, Oxford University, England
Eric Denkers, Cornell University
John Donelson, University of Iowa College of Medicine
Paul Englund, Johns Hopkins Medical School
Jean Feagin, Seattle Biomedical Research Institute
Frances Gillin, University of California, San Diego
Daniel Goldberg, Washington University
Richard K. Grenis, University of Manchester
Kasturi Haldar, Stanford University School of Medicine
Stephen Hoffman, Naval Medical Research Institute
Peter Hotez, Yale University
Richard Komuniecki, University of Toledo
John Mansfield, University of Wisconsin, Madison
James McKerrow, University of California, San Francisco
Tim Nilsen, Case Western Reserve University
Thomas Nutman, National Institutes of Health
Edward Pearce, Cornell University
Miercio Pereira, Tufts University
William Petri, University of Virginia
Steven Reed, Infectious Disease Research Institute
Steve Reiner, University of Chicago
David Roos, University of Pennsylvania
David Russell, Washington University Medical School
Alan Sher, National Institutes of Health
David Sibley, Washington University School of Medicine
Larry Simpson, University of California, Los Angeles
Sam Turco, University of Kentucky College of Medicine
C. C. Wang, University of California, San Francisco
Dyann Wirth, Harvard University

Course Coordinator

Robert Sabatini, University of Alabama, Birmingham

Course Assistant

Zachary Wood, University of Alabama, Birmingham

Students

Marion Doerner, University of Munich, Germany
Loïc Dupré, Institut Pasteur de Lille, France
Helen Faulkner, University of Manchester, England
Stenio Fragoso, Oswaldo Cruz Foundation, Brazil
Julie Gauthier, University of Maryland
Andrea Graham, Cornell University
Rhian Hayward, University of Oxford, England
Karl Hoffmann, Johns Hopkins University
Kirkwood Land, University of California, Los Angeles
Jeffrey Milner, University of Alabama, Birmingham
Yasuhiro Morita, Johns Hopkins University
Edward Nguu, University of Nairobi, Kenya
Harris Rotman, Thomas Jefferson University
Stepanka Vanacova, Charles University, Czechoslovakia
Lita Vieira, Hebrew University of Jerusalem, Israel
Stefan Wunsch, University of Würzburg, Germany

Embryology: Concepts and Techniques in Modern Developmental Biology

(June 13–July 27)

Directors

Eric H. Davidson, California Institute of Technology
Michael Levine, University of California, San Diego
David McClay, Duke University

Course Faculty

R. Andrew Cameron, California Institute of Technology
Sean Carroll, Howard Hughes Medical Institute
Wolfgang Driever, Massachusetts General Hospital
Alexander D. Johnson, University of California, San Francisco
James Posakony, University of California, San Diego
Nadia Rosenthal, Massachusetts General Hospital
Joel Rothman, University of Wisconsin
Noriyuki Satoh, Kyoto University, Japan
John Saunders, Jr., Marine Biological Laboratory
Martin Shankland, Harvard Medical School
Christopher C. Wylie, University of Minnesota School of Medicine
Janet Wylie, University of Minnesota School of Medicine

Teaching Assistants

Adina Bailey, University of California, San Diego
Scott Barolo, University of California, San Diego
Ashley Bruce, Harvard Medical School
Andres Collazo, California Institute of Technology
Joseph Corbo, University of California, San Diego
Steve Gendreau, University of Wisconsin
Gabrielle Kardon, Duke University
Carmen Kirchhamer, California Institute of Technology
Catherine Krull, California Institute of Technology
Catriona Logan, Duke University
Craig Neville, Massachusetts General Hospital
Ernesto Resnik, University of Minnesota Medical School
Denise Robb, University of Minnesota
Fabrizio Serluca, Massachusetts General Hospital

Lecturers

Doug Melton, Harvard University
Clifford Tabin, Harvard Medical School

Course Administrator

Jane Rigg, California Institute of Technology

Course Coordinator

Linda Huffer, Marine Biological Laboratory

Course Assistants

Allison Freeman, Wheaton College
Michael Rogers, New England College

Students

Fritz Aberger, University of Salzburg, Austria
André Adoutte, University of Paris, France
Hilrich Böger, Max-Planck-Institut, Germany
Christopher Brown, Vanderbilt University
Matthew Clark, Max-Planck-Institute, Germany
Sean Conner, Brown University
Laura Corley, Vanderbilt University
Anna Edlund, University of Virginia
Tamara Elul, University of California, Berkeley
Sacha Giardon, University of Basel, Switzerland
Brian Harfe, Carnegie Institution of Washington
Johanna Klerks, Utrecht University, The Netherlands
Rie Kusakabe, Kyoto University, Japan
Youn-Ho Lee, California Institute of Technology
David Lim, House Ear Institute
Eli Meir, University of Washington
Robert Peterson, Whitney Laboratory
Anna Laura Salvati, Istituto di Ricerche di Biologia Molecolare, Italy
Michael Shapiro, Harvard University Museum of Comparative Zoology
Guojun Sheng, The Rockefeller University
Jan Sumerel, University of North Carolina
Fiona Wardle, University College London, United Kingdom
Wei Weng, Columbia University
Darren Williams, Southampton University, United Kingdom
Yingzi Xue, Columbia University

Microbial Diversity (June 16–August 1)

Director

Edward Leadbetter, University of Connecticut
Abigail Salyers, University of Illinois

Faculty

Judith Armitage, University of Oxford, United Kingdom
Kurt Hanselmann, University of Zurich, Switzerland
Bruce Paster, Forsyth Dental Center

Teaching Assistants

Lars Damgaard, University of Aarhus, Denmark
David Graham, University of Illinois
Thomas Pitta, Rowland Institute for Science
Caroline Plugge, Wageningen Agricultural University, The Netherlands

Lecturers

Carl Bauer, Indiana University
Colleen Cavanaugh, Harvard University
Sharon Danielson, Rensselaer Polytechnic Institute
Jordan Konisky, University of Illinois, Urbana-Champaign
Terry Miller, New York State Department of Health
Hilary Morrison, Marine Biological Laboratory

Robert G. E. Murray, University of Western Ontario, Canada
 Sandra Nierzwicki-Bauer, Rensselaer Polytechnic Institute
 Gary Olsen, University of Illinois
 Nadja Shoemaker, University of Illinois
 Jeff Stein, Recombinant BioCatalysis, Inc.
 David Ward, Montana State University

Course Coordinator

Madeline Vargas, College of the Holy Cross

Course Assistant

Guido Tine, University of Connecticut

Students

Elena Barbieri, University of Urbino, Italy
 Richard Boone, University of Alaska
 Volker Bruchert, Indiana University
 James Comolli, Harvard University
 Jesse Dillon, University of Oregon
 Rachel Fernandes, University of Connecticut
 Ana Fernandez, Facultad de Quimica, Uruguay
 Christine Foreman, University of Toledo
 Daniel Gisi, Swiss Federal Institute of Technology, Switzerland
 Rachel Greedy, University of Leicester, United Kingdom
 Jay Gulledge, University of Alaska
 Mark Martin, Occidental College
 William Metcalf, University of Illinois
 Duane Moser, Center for Great Lakes Studies
 Jorge Olmos, La Universidad Nacional Autonoma, Mexico
 Jose Perez-Jimenez, University of Puerto Rico
 Stefan Ratering, Max-Planck-Institute, Germany
 Marcella Sackett, Indiana University
 Amy Schaefer, University of Iowa
 Annick Wilmette, Université de l'Etat à Liège, Belgium

Neural Systems & Behavior (June 16–August 9)

Directors

Janis Weeks, University of Oregon
 Harold Zakon, University of Texas

Faculty

Leon Avery, University of Texas Southwestern Medical Center
 Carol Barnes, University of Arizona
 Ronald L. Calabrese, Emory University
 Catherine Carr, University of Maryland
 Kathleen French, University of California, San Diego
 David Glanzman, University of California, Los Angeles
 Richard Hyson, Florida State University
 Masashi Kawasaki, University of Virginia
 William Kristan, University of California, San Diego
 Gilles Laurent, California Institute of Technology
 Richard Levine, University of Arizona
 Shawn Lockery, University of Oregon
 Michael Nusbaum, University of Pennsylvania School of Medicine
 Glen Prusky, University of Lethbridge, Canada
 William Roberts, University of Oregon
 Gary Rose, University of Utah
 Angela Wenning, Universität Konstanz, Germany

Teaching Assistants

Satoshi Amagai, University of Maryland
 Cecilia Armstrong, University of Oregon
 Dawn Blitz, University of Pennsylvania School of Medicine
 Shanning Chen, University of California, Los Angeles
 Richard Dyck, The Salk Institute
 Michael Ferrari, University of California, San Diego
 Matthew Friedman, Cornell University
 Jorge Golowasch, Brandeis University
 Rebecca Johnston, University of Arizona
 Maria Kubke, University of Maryland
 David Lenzi, University of Oregon
 Jane Lubischer, University of Oregon
 Lynne McAnelly, University of Texas
 Geoffrey Murphy, University of California, Los Angeles
 Kathryn Richards, Brandeis University
 Tom Smulders, Cornell University
 James Weimann, Stanford University
 Laura Wolszon, Columbia University

Lecturers

Geert De Vries, University of Massachusetts, Amherst
 Allison Doupe, University of California, San Francisco
 Stephen Highstein, Washington University
 Edward Kravitz, Harvard Medical School
 Rod Murphey, University of Massachusetts, Amherst

Course Coordinator

Evelyn Field, University of Lethbridge, Canada

Students

Srdjan Antic, Institute for Biological Research
 Mary Atkisson, Tufts University
 Thomas Bahl, Emory University
 Claire Balint, Boston University
 Amy Butler, University of Pennsylvania
 Lu Chen, University of Southern California
 Christofer Edwards, Columbia University
 Birgit Ehmer, University of Würzburg, Germany
 Edwin Gilliam, University of Arizona
 Jeanette Kuhn, Columbia University
 Brigitte Lavoie, Laval University, Canada
 John Layne, Duke University
 Greg Miller, Stanford University
 Pablo Monsivais, University of Washington
 Jonathan Pierce, University of Oregon
 Ilya Shekhter, Boston University
 Joseph Sisneros, Florida Institute of Technology
 Sheryl Starsinic, University of California, Davis
 Andrew Swensen, Brandeis University
 Harm Vitzthum, University of Regensburg, Germany

Neurobiology (June 16–August 17)

Directors

Gary Banker, University of Virginia Medical School
 Daniel Madison, Stanford University Medical Center

Course Faculty

Mark Bennett, University of California, Berkeley
 Ann Marie Craig, University of Illinois

Donald Faber, Medical College of Pennsylvania
Craig Garner, University of Alabama, Birmingham
John Heuser, Washington University School of Medicine
Stephen Jones, Case Western Reserve University
Maurice Kernan, State University of New York, Stony Brook
Maurine Linder, Washington University School of Medicine
Diane Lipscombe, Brown University
David Ogden, National Institute for Medical Research, London
Alberto Pereda, Medical College of Pennsylvania
Thomas Reese, National Institutes of Health
Robert Rosenberg, University of North Carolina
Morgan Sheng, Howard Hughes Medical Institute
Carolyn Smith, National Institutes of Health
Stephen Smith, Stanford University School of Medicine
Mark Terasaki, University of Connecticut Health Center
Stuart Thompson, Stanford University
Ginger Withers, University of Virginia
Susan Wray, National Institutes of Health

Lecturers

Michael Greenberg, Children's Hospital
Stanley B. Kater, University of Utah School of Medicine
Arthur Konnerth, University of Saarland, Germany
Angus Nairn, The Rockefeller University
Nelson Spruston, Northwestern University
Joshua Zimmerberg, National Institutes of Health

Course Assistants

Eleanore Edson, Stanford University
Muffie Fulton, Brown University

Students

Veronica Alvarez, University of Buenos Aires, Argentina
Celine Auger, Max-Planck-Institut, Germany
Shaowen Bao, University of Southern California
Bruce Cohen, University of California, Berkeley
Shari Gelber, Columbia University
Paul Gray, University of California, Los Angeles
Kristina Micheva, Université de Montréal, Canada
Dimitar Nikolov, Sloan-Kettering Institute
Atsumi Nitta, Gifu Pharmaceutical University, Japan
Venugopala Reddy, Tata Institute of Fundamental Research, India
Caitlin Smith, Yale University
Daniel Stimson, University of Arizona

Physiology: Cellular and Molecular Biology

(June 16–July 27)

Directors

Kerry Bloom, University of North Carolina, Chapel Hill
Mark Mooseker, Yale University

Course Faculty

William Bement, University of Wisconsin
Ruth Empson, University of Oxford, United Kingdom
Antony Galione, University of Oxford, United Kingdom
Sally Kornbluth, Duke University Medical Center
Daniel Lew, Duke University Medical Center
Jennifer Lippincott-Schwartz, National Institutes of Health
Robert E. Palazzo, University of Kansas
Edward Salmon, University of North Carolina, Chapel Hill
Roger D. Sloboda, Dartmouth College
Katherine Swenson, Duke University Medical Center

Edwin Taylor, University of Chicago
Mark Terasaki, University of Connecticut Health Center
Joseph S. Wolenski, Yale University

Teaching Assistants

Elaine Bardes, Duke University Medical Center
Tama Hasson, Yale University
Clay Lyddane, University of Kansas
Valerie Mermall, Yale University
John Presley, National Institutes of Health
Samara Reck-Peterson, Yale University
Jennifer Waters, University of North Carolina, Chapel Hill
Rebekah White, Duke University Medical Center
Charlie Yang, University of North Carolina, Chapel Hill
Jing Yang, Duke University Medical Center
Sam Yang, University of North Carolina, Chapel Hill
Elaine Yeh, University of North Carolina, Chapel Hill

Lecturers

Steven Block, Princeton University
George Bloom, University of Texas
Tatsuya Hirano, Cold Spring Harbor Laboratory
Elizabeth Luna, Worcester Foundation for Biomedical Research
Jonathan Pines, Wellcome/CRC Institute, United Kingdom
George Vande Woude, Frederick Cancer Research & Development Center
Curt Wittenberg, The Scripps Research Institute
Tim Yen, Fox Chase Cancer Center

Course Assistants

Caroline Day, Yale University
Noelle Frey, Yale University

Students

Vivek Abraham, Carnegie Mellon University
Vishwas Agashe, National Center for Biological Science, Germany
Birke Bartosch, Imperial Cancer Research Fund, United Kingdom
James Bingham, Johns Hopkins University
Matteo Caleo, University of Pisa, Italy
Lisa Cameron, Stanford University
Magdalena Chrzanowska-Wodnicka, University of North Carolina, Chapel Hill
Bryan Clubb, University of Western Ontario, Canada
Stephanie Connors, University of Pennsylvania
Brian Fiske, University of Virginia
Laura Ginkel, University of Washington
Dawn Grant, Louisiana State University Medical Center
Kurt Hankenson, Purdue University
Sophie Janssens, Janssen Research Foundation, Belgium
James Jontes, Scripps Research Institute
Oxana Karpenko, Brown University
Brett Kaufman, Southwestern Medical Center
Jennifer King, Duke University
Peter Koulen, Max-Planck-Institute, Germany
Shauna Kubose, Albert Einstein College of Medicine
Edward Lobenhofer, Duke University
John Mitchem, University of Bristol, United Kingdom
Laura Mitic, Yale University
Pedro Santiago, University of Puerto Rico
Bradley Schnackenberg, University of Kansas
Nancy Searby, Stanford University
Michal Shtenberg-Blumenfeld, Technion, Israel
Anna Sokac, University of Wisconsin, Madison

Charlene Stern, Yale University
 Elizabeth Stillwell, Emory University
 Sonya Summerour, University of California, San Diego
 Masahiko Tanaka, Fujita Health University, Japan
 Leana Topper, University of Virginia
 Arpita Upadhyaya, University of Notre Dame
 Amber Wells, University of Pennsylvania
 Adele Wright, Georgia Institute of Technology

Siobhán McClean, Elan Corporation Research Institute, Ireland
 Paul Meechan, Merck Research Laboratories
 Roger Oda, Allergan, Inc.
 Carmine Pariente, Emory University
 Donald Phipps, Jr., Orange County Water District
 Toby Velt, Harvard Medical School
 Sam Ward, University of Arizona
 John Zhang, University of Chicago
 Ofer Zohar, National Institutes of Health

Short Courses

Analytical & Quantitative Light Microscopy (May 9–May 17)

Directors

Greenfield Sluder, Worcester Foundation for Biomedical Research
 David Wolf, Worcester Foundation for Biomedical Research

Faculty

William B. Amos, Medical Research Council, United Kingdom
 Hagan Bayley, Worcester Foundation for Biomedical Research
 Richard Cardullo, University of California, Riverside
 Jeff Gelles, Brandeis University
 Shinya Inoue, Marine Biological Laboratory
 Edward Salmon, University of North Carolina, Chapel Hill
 Randi Silver, Cornell University Medical College
 Kenneth Spring, National Institutes of Health
 Yu-li Wang, Worcester Foundation for Biomedical Research

Teaching Assistants

Christine McKinnon, Worcester Foundation for Biomedical Research
 Elizabeth Thompson, Worcester Foundation for Biomedical Research

Lecturer

Rudolf Oldenbourg, Marine Biological Laboratory

Course Coordinator

Frederick Miller, Worcester Foundation for Biomedical Research

Students

Michael Ahrens, State University of New York, Stony Brook
 Takashi Akiyama, Yokogawa Electric Corp., Japan
 Nihal Altan, The Rockefeller University
 Francisco Alvarez-Leef, University of Texas Medical Branch
 Ahmed Bouzid, Noran Instruments, Inc.
 John Cordingley, Merck Research Laboratories
 Noemi Fernandez, Florida International University
 Maria Fischer, Friedrich Miescher Institute, Switzerland
 Martin Gluch, Carl Zeiss, Inc., Germany
 Kimberly Goslin, Harvard Medical School
 Tamara Grant, Louisiana State University Medical Center
 Andrew Greenberg, Tufts University
 Earle Holmes, Loyola University
 Han Htun, National Cancer Institute
 David Keefe, Yale University School of Medicine
 David Krizaj, Beckmann Vision Center
 David Leaf, Western Washington University
 Robert Liburdy, Lawrence Berkeley Laboratory

Fundamental Issues in Vision Research (August 11–August 24)

Director

David Papermaster, University of Texas Health Science Center

Faculty

Robert Barlow, State University of New York Health Science Center
 Robert W. Baughman, National Institutes of Health
 Roy Beck, Jacob Center for Health Research, Inc.
 David C. Beebe, Washington University School of Medicine
 Eliot Berson, Massachusetts Eye & Ear Institute
 Connie Cepko, Harvard University Medical School
 Max Cynader, University of British Columbia, Canada
 Dusanka Deretic, Kellogg Eye Center
 Kay Dickersin, University of Maryland
 John E. Dowling, Harvard University
 Frederick Ferris, National Institutes of Health
 Benjamin Geiger, Weizmann Institute of Science, Israel
 Daniel A. Goodenough, Harvard Medical School
 Jonathan Horton, University of California, San Francisco
 Joseph Horwitz, University of California, Los Angeles
 Carl Kupfer, National Institutes of Health
 Ellen Liberman, National Institutes of Health
 Robert Malchow, University of Illinois
 Richard Masland, Massachusetts General Hospital
 Jeremy Nathans, Johns Hopkins University School of Medicine
 Robert Nussenblatt, National Institutes of Health
 Krzysztof Palczewski, University of Washington
 Joram Piatigorsky, National Institutes of Health
 Haohua Qian, Harvard University
 Nancy Ransom, University of Texas Health Science Center
 Elio Raviola, Harvard Medical School
 Dwight Stambolian, University of Pennsylvania
 Tung-Tien Sun, New York University Medical Center
 Paul Wasson, Eye Health Services

Lecturers

Dean Bok, University of California, Los Angeles
 Ron DePinho, Albert Einstein College of Medicine
 M. Elizabeth Fini, New England Medical Center
 Paul Kaufman, University of Wisconsin
 Joseph O'Tousa, University of Notre Dame
 Harris Rippes, University of Illinois

Course Assistant

Michael Rogers, New England College

Students

Bassem Bejjani, Baylor College of Medicine
 Tiera Coston, Tulane University

Diane Darland, Oregon Health Sciences University
Xiaohua Gong, Scripps Research Institute
Beth Hinkle, University of California, Santa Barbara
Juliette Johnson, University of California, Los Angeles
Camille King, National Institutes of Health
Andria Lee, Scripps Research Institute
Richard Lee, University of Miami School of Medicine
Jennifer Lin-Jones, University of Washington
Nan Min, Cornell University
Royce Mohan, Tufts University
Deborah Otteson, University of Michigan
Brooke Sanger, University of Kansas Medical Center
Daniel Stamer, University of Arizona
David Stockton, Baylor College of Medicine
Jeffrey Toy, Johns Hopkins University
Valerie Wallace, University College London, United Kingdom
Ruey-Bing Yang, University of Texas Southwestern Medical

Jayshiro Tashiro, Northern Arizona University
Michele Tennant, University of Florida
Timothy Unga, U.S. Coast Guard
Richard Winant, State University of New York

Methods in Computational Neuroscience ***(August 4–August 31)***

Directors

David Kleinfeld, University of California, San Diego
David Tank, AT&T Bell Laboratories

Faculty

Lawrence Abbott, Brandeis University
Moshe Abeles, The Hebrew University of Jerusalem, Israel
William Bialek, NEC Research Institute
Carol Colby, National Eye Institute
Kerry Delaney, Simon Fraser University, Canada
Alain Destexhe, Laval University School of Medicine, Canada
Bard Ermentrout, University of Pittsburgh
Kevin Fox, University of Wales, Cardiff, United Kingdom
David Hansel, Ecole Polytechnique, France
Roderick Jensen, Wesleyan University
Daniel Johnston, Baylor College of Medicine
Nancy Kanwisher, Harvard University
Nancy Kopell, Boston University
Terry Kovacs, AT&T Bell Laboratories
Rodolfo Llinás, New York University Medical Center
Kevin Martin, University of North Carolina, Chapel Hill
Partha Mitra, AT&T Bell Laboratories
John Rinzel, National Institutes of Health
Terrance Sejnowski, The Salk Institute
H. Sebastian Seung, AT&T Bell Laboratories
Arthur Sherman, National Institutes of Health
Karen Sigvardt, University of California, Berkeley
Frederick Sigworth, Yale School of Medicine
William Skaggs, University of Arizona
David Somers, Massachusetts Institute of Technology
Haim Sompolsky, The Hebrew University of Jerusalem, Israel
David Sparks, University of Pennsylvania
Karel Svoboda, AT&T Bell Laboratories
Naftali Tishby, The Hebrew University of Jerusalem, Israel
Roger Traub, IBM
Michael Vanier, California Institute of Technology
Xiao-Jing Wang, Brandeis University
Ralf Wessel, University of California, San Diego
John White, Boston University
Steven Zucker, McGill University, Canada

Medical Informatics (May 29–June 5)

Director

Daniel Masys, University of California, San Diego

Faculty

Paul Clayton, Columbia University
George Hripcsak, Columbia Presbyterian Medical Center
Stephen Johnson, Columbia Presbyterian Medical Center
Lawrence Kingsland, National Library of Medicine
David Landsman, National Library of Medicine
Donald D.A.B. Lindberg, National Library of Medicine
Carol Newton, University of California School of Medicine,
Los Angeles
Rick Rodgers, National Library of Medicine
Jay Sanders, Global Telemedicine Group

Students

Paul Antonecchia, St. Vincent's Medical Center
Cody Arnold, Harris Methodist Hospital
Steven Bengelsdorf, Boston University Medical Center
Emmanuel Besa, Medical College of Pennsylvania
Frances Brahmi, Indiana University School of Medicine
James Brassel, St. Clare's Hospital
Karen Brown, Yale New Haven Hospital
Cynthia Cappello, Naval Medical Center San Diego
Mary Ellen Cunningham, St. Vincent's Medical Center
Brad Cushing, University of Maryland School of Medicine
Adrienne Fleckman, Beth Israel Medical Center
Margarita Gonzalez, University of Puerto Rico
Catherine Graber, University of Illinois, Chicago
Robert Grealish, Forbes Health System
Jacob Jones, Tri County Medical College of Virginia
James Judge, University of Connecticut
Douglas Knittel, National Naval Medical Center
Scott Luria, University of Vermont
Laura Mandel, State University of New York Health Science Center
Brian Mavis, Michigan State University
Faith McGrath, Yale University School of Medicine
John Pollock, Crozer-Keystone Health System
Ron Poropatich, Walter Reed Army Medical Center
Margaret Richwine, Indiana University School of Medicine
Albert Salas, Association of American Medical Colleges
Candace Scales, Howard University

Teaching Assistants

Howard Eichenbaum, Boston University
David McCormick, Yale University School of Medicine
Robert Shapley, New York University

Lecturers

Allison Doupe, University of California, San Francisco
John Hopfield, California Institute of Technology
Stephen Kosslyn, Harvard University

Course Assistants

Gabby Martinez, Mount Holyoke College
Rob de Ruyter Van Steveninck, NEC Research Institute

Students

Terry Alkasab, Tufts University School of Medicine
 Amir Assadi, University of Wisconsin, Madison
 Steve Baccus, University of Miami
 Parveen Bawa, Simon Fraser University, Canada
 Janet Casagrand, University of Colorado
 Rudi DeKoker, Stanford University
 Valentin Dragoi, Duke University
 Elrich Egert, University of Tübingen, Germany
 Jaime Eugenin, University of Santiago, Chile
 Tom Ferrée, University of Oregon
 Raj Gandhi, University of California, San Francisco
 Andreas Herz, University of Bremen, Germany
 Anton Krukowski, University of California, San Francisco
 Boris Lamotte, Laboratoire de Physiologie et Biologie de la Motricité, France
 Mickey London, Hebrew University, Israel
 Anita Rado, University of Arizona
 Wouter-Jan Rappel, Northeastern University
 Aeyal Raz, Hebrew University Hadassah Medical School, Israel
 Christie Seay, University of Texas
 Krishna Shenoy, California Institute of Technology
 Gregory Smith, University of California, David
 Fred Soo, Harvard University
 Areti Tzelepi, State University of New York

Microinjection Techniques in Cell Biology**(May 21–May 28)***Director*

Robert B. Silver, Marine Biological Laboratory

Faculty

Suzanne Klaessig, Cornell University
 Douglas Kline, Kent State University
 Paul McNeil, Medical College of Georgia
 Gernot Presting, Cornell University
 Eric Shelden, University of Michigan

Course Assistants

William Fripp, Marine Biological Laboratory
 Lisa Mehlmann, Kent State University

Students

John Brumell, Hospital for Sick Children, Canada
 JoAnn Castelli, George Washington University
 Michael Chastain, Merck Research Laboratories
 Patricia Clissold, University of Leeds, United Kingdom
 Mark Clymer, Rhone-Poulenc Rorer
 Barry Kaplan, University of Pittsburgh School of Medicine
 Thomas Krarup, Copenhagen University, Denmark
 Janet Lewis, University of Virginia
 Timothy Lyerla, Clark University
 Alfred May, National Institutes of Health
 Nicole McNaughton, Medical Research Council, United Kingdom
 Sanjay Pimplikar, Case Western Reserve University
 Gerard Ramakers, Netherlands Institute for Brain Research,
 The Netherlands
 Rosario Reyes, University of Oregon
 Ilija Rivera-Rivera, University of Illinois
 Andrey Shaw, Washington University

Sandra Souza, Tufts University
 Bayar Thimmapaya, Northwestern University
 Joe Tietge, U.S. Environmental Protection Agency

Neurobiology & Development of the Leech**(August 11–August 31)***Director*

Pierre Drapeau, Montreal General Hospital Research Institute,
 Canada
 Martin Shankland, Harvard Medical School

Faculty

Andreas Baader, University of Zurich, Switzerland
 Shirley Bissen, University of Missouri
 Salvatore Carbonetto, Montreal General Hospital, Canada
 Francisco Fernandez de Miguel, Universidad Nacional Autonoma,
 Mexico
 John Jellies, Western Michigan University
 Jorgen Johansen, Iowa State University
 Anna Kleinhaus, New York Medical College
 William Kristan, University of California, San Diego
 Mark Martindale, University of Chicago
 Barbara Modney, Cleveland State University
 Kenneth Muller, University of Miami School of Medicine
 John Nicholls, University of Basel, Switzerland
 Christine Sahley, Purdue University
 Catherine Wedeen, New York Medical College

Course Assistant

Jennifer Nixon, Marine Biological Laboratory

Students

Michael Baker, Columbia University
 Crista Barberini, R.S. Dow Neurological Sciences Institute
 Brian Burrell, Purdue University
 Jing Fang, Yale University School of Medicine
 Barbara Foerch, Duke University
 Elana Levantini, Università degli Studi di Pisa, Italy
 Mark Masino, Emory University
 Jorge Pérez-León, Instituto de Fisiología Celular, Mexico
 Ravi Ranjan, University of Pennsylvania School of Medicine
 José Sotelo, Universidad de la Republica, Uruguay
 Shigeo Watanabe, University of Tokyo, Japan
 Xian Zhang, New York Medical College

Optical Microscopy and Imaging in the Biomedical Sciences (October 23–October 30)*Director*

Colin Izzard, State University of New York, Albany

Faculty

Joseph DePasquale, New York State Department of Health
 Robert Hard, State University of New York, Buffalo
 Brian Herman, University of North Carolina, Chapel Hill
 Frederick Maxfield, Cornell University Medical College
 John Murray, University of Pennsylvania
 Kenneth Spring, National Institutes of Health
 Jason Swedlow, University of California, San Francisco

Teaching Assistants

Richik Ghosh, Columbia University College of Physicians
& Surgeons
Wade Sigurdson, State University of New York, Buffalo
Elizabeth Welnhof, State University of New York, Buffalo

Lecturers

Jan Hinsch, Leica, Inc.
Shinya Inoue, Marine Biological Laboratory
H. Ernst Keller, Carl Zeiss, Inc.
Rudolf Oldenbourg, Marine Biological Laboratory
Martin Scott, Consultant in Scientific Imaging

Course Assistant

Michael Rogers, Marine Biological Laboratory

Students

Ariel Agmon, West Virginia University
Agustin Alconana, European Molecular Biology Laboratory,
Germany
Per Arkhammar, BiolImage Novo Nordisk, Denmark
Douglas Bannerman, University of Maryland
Leif Eriksson, Uppsala Generic Center, Sweden
Charles Filburn, National Institute on Aging
William Flynn, State University of New York, Buffalo
Craig Gelband, University of Florida
Dwayne Godwin, State University of New York, Stony Brook
Masako Isokawa, University of California, Los Angeles
Kaoru Katoh, Marine Biological Laboratory
Carl Thomas Korte, National Institutes of Health
Olga Kovbasnjuk, National Institutes of Health
Dianne Kube, Case Western Reserve University
Djokolngar Maouyo, Johns Hopkins University
Wendy Morse, University of North Carolina, Chapel Hill
Barbara Muller-Borer, University of North Carolina, Chapel Hill
Isabel Munoz-Barro, National Institutes of Health
Ilya Plonsky, National Institutes of Health
John Roths, Texas A&M University
Ole Skygebjerg, BiolImage Novo Nordisk, Denmark
Elizabeth Stryjewski, NASA/Dynamac
Livingston Van De Water, Shriners Burn Institute
Teng Wu, Vanderbilt University

Rapid Electrochemical Measurements in Biological Systems (August 16–August 21)

Director

Greg Gerhardt, University of Colorado Health Sciences Center

Teaching Assistants

Scot Brock, University of Colorado Health Science Center
Wayne Cass, University of Kentucky
Lynette Daws, University of Texas Health Sciences Center
Alain Gratton, McGill University, Canada
Meleik Hebert, University of Colorado Health Science Center
Alexander Hoffman, University of Colorado Health Science Center
Scott Robinson, University of Colorado Health Science Center
Steven Robinson, University of Colorado Health Science Center

Course Coordinator

Debbie Ristow, University of Colorado Health Science Center

Students

Jan Acworth, Massachusetts College of Pharmacy
Emine Babar, Cukurova University, Turkey
Jian-Xin Bao, Columbia University
Juliana Brooks, Berkshire Laboratories
Clint Buchanan, Medical College of Georgia
Jonathan Carlson, Loma Linda University
Victor Chen, Brooklyn Veterans Administration Medical Center
Edward Cutrell, University of Oregon
Shelly Dickinson, University of Colorado Health Sciences Center
Linda Dwsokin, University of Kentucky
Gregory Ford, Meharry Medical College
Brooks Gentry, University of Arkansas for Medical Sciences
Phillip Heller, National Institute on Aging
Hua Liu, Loma Linda University
Timothy Maher, Massachusetts College of Pharmacy
Al Ngai, University of Washington
Yoshiaki Omura, Heart Disease Research Foundation
Michael Owens, University of Arkansas for Medical Sciences
John Panos, University of Wisconsin, Milwaukee
Joel Prokisch, University of Arkansas for Medical Science
Ian Pullar, Lilly Research Centre Limited, United Kingdom
Lynne Samuelson, U.S. Army Natick
Yvonne Schmitz, New York University Medical Center
Nitish Thakor, Johns Hopkins Medical School

Workshop on Molecular Evolution (August 4–August 16)

Directors

Daniel B. Davison, University of Houston
Mitchell Sogin, Marine Biological Laboratory

Faculty

Mary Berbee, University of British Columbia, Canada
W. Ford Doolittle, Dalhousie University, Canada
Joseph Felsenstein, University of Washington
David Hillis, University of Texas
Mike Holder, University of Houston
Lee Hood, University of Washington
Gerald Joyce, The Scripps Research Institute
David Maddison, University of Arizona
Roger Milkman, University of Iowa
Michael Miyamoto, University of Florida
Lisa Nagy, University of Arizona
David Nelson, Gaylor College of Medicine
Gary Olsen, University of Illinois
William Pearson, University of Virginia
Rudolf Raff, Indiana University
Michael Rice, University of Houston
Margaret Riley, Yale University
Monica Riley, Marine Biological Laboratory
David Swofford, Smithsonian Institution
Bruce Walsh, University of Arizona

Teaching Assistants

Janet Siefert, University of Houston
Steven Thompson, Washington State University

Lecturer

David Wheeler, Baylor College of Medicine

Course Assistants

Elizabeth Gale, University of Pennsylvania Medical School
 Jennie Halfant, University of Virginia

Students

J. Ewann Agenbroad, Woods Hole Oceanographic Institute
 Tasha Altheide, University of Arizona
 Brian Arbogast, Wake Forest University
 Jon Ashen, University of California, Santa Cruz
 Luis Cadavid, University of Wisconsin Primate Center
 Robert Campbell, Rutgers Ares Advanced Technology
 Page Caufield, University of Alabama, Birmingham
 Estella Chen, Emory University
 Susan Cohen, George Mason University
 David Coleman, University of Oxford, United Kingdom
 Scott Dawson, University of California, Berkeley
 Jose Farfan, Colorado State University
 Jennifer Grenier, University of Wisconsin, Madison
 Pamela Groves, University of Alaska
 Laura Gutierrez, University of Houston
 John Dawdon, Yale University School of Medicine
 Robert Hirt, Natural History Museum, London, United Kingdom

Tamara Horton, Princeton University
 Gwendolyn Howze, Texas Southern University
 Johannes Jehle, Wageningen Agricultural University, The Netherlands
 Marcia Kalish, Centers for Disease Control and Prevention
 Richard Kessin, Columbia University
 Gretchen Kuldau, University of Kentucky
 Nikos Kypides, University of Illinois, Champaign-Urbana
 Eric Linton, Rutgers University
 Thomas McNeill, University of Houston
 Monty Montano, Harvard University
 Enrique Morett, University of Mexico, Mexico
 Lorraine Olendzenski, University of Connecticut
 Kathleen Page, Southern Oregon State College
 Oris Sanjur, Rutgers University
 Jorge Santiago-Blay, University of Chicago
 Riitta Savolainen, University of Helsinki, Finland
 Inder Sacena, University of Texas, Austin
 Konstantin Severinov, The Rockefeller University
 David Smith, Williams College
 Jeffrey Villinski, Indiana University, Bloomington
 Regina Wetzer, University of South Carolina
 Heather Wilkinson, University of Kentucky
 Charles Wimpee, University of Wisconsin, Milwaukee

Summer Research Programs



Principal Investigators

Alkon, Daniel L., National Institutes of Health
Armstrong, Clay, University of Pennsylvania
Armstrong, Peter B., University of California, Davis
Augustine, George J., Duke University Medical Center
Barlow, Jr., Robert B., State University of New York
Health Science Center
Basil, Jennifer, Marine Biological Laboratory
Beaugé, Luis, Instituto M. y M. Ferreyra, Argentina
Behbehani, Michael, University of Cincinnati
Belanger, Jim, University of Arizona
Bennett, Michael V. L., Albert Einstein College of Medicine
Berlin, Joshua, Bockus Research Institute
Bingham, Eula, University of Cincinnati
Boal, Jean Geary, University of Texas Medical Branch
Bodznick, David, Wesleyan University
Boron, Walter F., Yale University Medical School
Borst, David, Illinois State University
Boyer, Barbara, Union College
Boyle, Mary, La Jolla Cancer Research Foundation
Brady, Scott T., The University of Texas Southwestern
Medical Center, Dallas
Brown, Joel E., Albert Einstein College of Medicine
Browne, Carole, Wake Forest University
Burger, Max M., Friedrich Miescher Institut, Switzerland
Cai, Wei-Jun, University of Georgia
Cardell, Robert R., University of Cincinnati
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Knudson, Robert, Instrumental Development Engineer
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Maccaro, Jackie, Laboratory Assistant

Visiting Investigators

Arimoto, Rieko, Nikon Corporation, Tokyo, Japan
Hiramoto, Yukio, University of the Air, Chiba, Japan
Inoué, Theodore D., Universal Imaging Corporation, West Chester, PA
Suzuki, Keisuke, Olympus Corporation, Hachioji, Japan
Oosawa, Fumio, Aichi Institute of Technology, Toyota, Japan, and
PRESTO of JRDC, Keihanna, Japan
Otaki, Tatsuro, Nikon Corporation, Tokyo, Japan
Tran, Phong, University of North Carolina, Chapel Hill
Yamada, Katsuyuki, Nagoya University, Nagoya, Japan

The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution

Major emphasis in the Bay Paul Center for Comparative Molecular Biology and Evolution is placed upon comparative and phylogenetic studies of genes and genomes, molecular microbial ecology and biodiversity and evolution of host defense mechanisms in marine invertebrates. The Center encourages studies of genotypic diversity across all phyla and promotes the use of modern molecular genetics and phylogeny to gain insights into the evolution of molecular



structure and function. The Marine Biological Laboratory has considerable strength in comparative molecular biology and evolution including Mitchell Sogin's studies of molecular evolution in eukaryotes, Monica Riley's metabolic database and evolutionary studies of protein sequences, Neal Cornell's comparative molecular studies of genes critical to heme biosynthesis, and Norman Wainwright's studies of host defense mechanisms in marine invertebrates.

Other collaborative projects include studies of P540 evolution (M. Sogin and John Stegeman's laboratory at Woods Hole Oceanographic Institution [WHOI]); a molecular ecology component of the Long Term Ecological Research project (M. Sogin's laboratory and John Hobbie of the Ecosystems Center); and studies of molecular diversity among marine protists and bacteria (with marine microbiologists and WHOI). Future recruiting efforts will focus upon molecular evolution in developmental biology and genome sciences.

The Center has excellent resources for studies of molecular evolution: automated DNA sequencing, well-equipped research laboratories, and powerful computational facilities. In addition to participating in the Parasitology and Microbial Diversity courses, the Center sponsors the Workshop in Molecular Evolution at the MBL, which has gained an international reputation for excellence.

Resident Core Investigators

Sogin, Mitchell, Director and Senior Scientist
Cornell, Neal, Senior Scientist
Riley, Monica, Senior Scientist
Wainwright, Norman, Senior Scientist

Laboratory of Neal Cornell

Research in this laboratory is concerned with the comparative molecular biology of genes that encode the enzymes for heme biosynthesis, with particular emphasis on 5-aminolevulinic synthase, the first enzyme in the pathway. Because the ability to produce heme from common metabolic materials is a near universal requirement for living organisms, these genes provide useful indicators of molecular aspects of evolution. For example, 5-aminolevulinic synthase in vertebrate animals and simple eukaryotes such as yeast and *Plasmodium falciparum* have high sequence similarity to the enzyme from the alpha-purple subgroup of eubacteria. This supports the suggestion that the ancestor of mitochondria in these eukaryotes and animals may have been a member of that group of bacteria. The analysis also raises the possibility that plant and animal mitochondria had different origins. Future work will focus on characterizing the aminolevulinic synthase genes of invertebrates and simple eukaryotes in addition to the ones mentioned above. Other studies in the laboratory have been concerned with the effects of environmental pollutants on heme biosynthesis in marine fish, and it has been shown

that polychlorinated biphenyls (PCBs) enhance the expression of the gene for aminolevulinic synthase.

Staff

Cornell, Neal W., Senior Scientist
Dunlap, Rachel, Research Associate
Faggart, Maura A., Research Assistant
Macarro, Jackie, Laboratory Assistant

Visiting Scientist

Fox, T. O., Harvard Medical School

Laboratory of Norman Wainwright

The mission of the laboratory is to understand the molecular defense mechanisms exhibited by marine invertebrates in response to invasion by bacteria, fungi, and viruses. The primitive immune systems demonstrate unique and powerful strategies for survival in diverse marine environments. The key model has been the horseshoe crab *Limulus polyphemus*. *Limulus* hemocytes exhibit a very sensitive LPS-triggered protease cascade which results in blood coagulation. Several proteins found in the hemocyte and hemolymph display microbial binding proteins that contribute to antimicrobial defense. Commensal or symbiotic microorganisms may also augment the antimicrobial mechanisms of macroscopic marine species. Secondary metabolites are being isolated from diverse marine microbial strains in an attempt to understand their role. Microbial participation in oxidation of the toxic gas hydrogen sulfide is also being studied.

Staff

Wainwright, Norman, Senior Scientist
Barry, Kevin, Research Assistant
Child, Alice, Research Assistant
Kreiling, Jill, Postdoctoral Research Associate

Visiting Investigator

Anderson, Porter, University of Rochester

Molecular Evolution of Genomes

The genome of the bacterium *Escherichia coli* contains all of the information required for a free-living chemoautotrophic organism to live, adapt, and multiply. The information content of the genome can be dissected from the point of view of understanding the role of each gene and gene product in achieving these ends. The many functions of *E. coli* have been organized in a hierarchical system representing the complex physiology and structure of the cell. In collaboration with Dr. Peter Karp of SRI International, an electronic encyclopedia of information is being constructed on the genes, enzymes, metabolism, transport processes, regulation, and cell structure of *E. coli*. The interactive EcoCyc program is now publicly available and has graphical hypertext displays, including literature citations, on nearly all of *E. coli* metabolism, all genes and their locations, a hierarchical system of cell functions and some regulation processes. This work is continuing.

In addition, the *E. coli* genome contains valuable information on molecular evolution. We are analyzing the sequences of proteins of *E. coli* in terms of their evolutionary origins. By grouping like sequences and tracing back to their common ancestors, one learns not only about the paths of evolution for all contemporary *E. coli* proteins, but one extends even further back before *E. coli*, traversing millennia to

the earliest evolutionary times when a relatively few ancestral proteins served as ancestors to all contemporary proteins of all living organisms. The nearly complete genome sequence of *E. coli* and sophisticated sequence analysis programs permit us to identify evolutionary related protein families, determining ultimately what kinds of unique ancestral sequences generated all of present-day proteins. The data developed in the work has proved to be valuable to the community of scientists sequencing microbial genomes. *E. coli* data serve as needed reference points.

Staff

Riley, Monica, Senior Scientist
Crossin, Glenn, Research Assistant I
Pelligriini-Toole, Alida, Research Assistant II

Program in Comparative Molecular Biology and Evolution

The Program in Comparative Molecular Biology and Evolution employs comparative phylogenetic studies of genes and genomes to define patterns of evolution that gave rise to contemporary biodiversity on the planet Earth. We are especially interested in discerning how the eukaryotic cell was invented as well as the identity of microbial groups that were ancestral to animals, plants, and fungi. We take advantage of the extraordinary conservation of ribosomal RNAs to define phylogenetic relationships that span the largest of evolutionary distances. These studies have overhauled traditional eukaryotic microbial classification systems. We have discovered new evolutionary assemblages that are as genetically diverse and complex as plants, fungi, and animals. The nearly simultaneous separation of these eukaryotic groups (described as the eukaryotic "Crown") occurred approximately one billion years ago and was preceded by a succession of earlier diverging protist lineages, some as ancient as the separation of the prokaryotic domains. At the same time this database provides a powerful tool for the newly emerging discipline of molecular ecology. Using the ribosomal RNA database and nucleic acid-based probe technology, it is possible to detect and monitor microorganisms including those that cannot be cultured in the laboratory. This strategy has revealed new habitats and major revelations about geographical distribution of microorganisms.

More recently we have initiated a program to sample genomic diversity from eukaryotic microorganisms that do not have mitochondria. We previously demonstrated that these taxa represent some of the earliest diverging lineages in the evolutionary history of eukaryotes. The objective is to develop a set of additional molecular markers for studying molecular evolution. These will be invaluable in unraveling sudden evolutionary radiations that cannot be resolved by rRNA comparisons and will provide insights into the presence or absence of important biochemical properties in the earliest ancestors common to all eukaryotic species.

Staff

Sogin, Mitchell L., Director and Senior Scientist
Hinkle, Greg, Postdoctoral Research Associate
Morrison, Hilary G., Postdoctoral Research Associate
Silberman, Jeffrey, Postdoctoral Research Associate

Visiting Investigators

Bahr, Michele, Ecosystems Center
Barnhisel, Rae, Postdoctoral Sloan Fellow
Collins, Allen, University of California, Berkeley

BioCurrents Research Center

A part of the NIH National Center for Research Resources biomedical technology program, the BioCurrents Research Center (the successor to the National Vibrating Probe Facility) has pioneered methods in the study of transmembrane currents and, over the years since its establishment at the MBL in 1983, has hosted diverse scientific endeavors. The BioCurrents Research Center (BRC), like other Biomedical Research Technology Program centers, provides visiting investigators access to a variety of unique technologies as well as new approaches to experimentation in the biomedical sciences.

All the probe technologies developed at the BRC are based on the principles of a self-referencing electrode maximizing sensitivity by noise and drift reduction. All the probes are non-invasive and generally placed in close proximity to the membrane of cells or tissues, in some cases at submicron distances. Four systems are available or being developed at the BRC. The oldest, the current probe developed in 1974, is still available for the study of external current densities resulting from the general net balance of ion transport. Most demand is for the ion-selective probes, which look at the transmembrane transport of specific ions such as calcium, potassium and protons. A most important feature of this system is its ability to detect non-electrogenic transporters. Two additional systems are now being developed, the polarographic probes and the BioKelvin probes. The former uses the chemical characteristics of molecules under applied voltages to detect their local flux. Oxygen measurement is being used to pilot this technology. The BioKelvin probe is a direct extension of physical principles used in materials science but extensively adapted to biological systems. It operates in air. This work, done in close collaboration with Dr. Iain Baikie of Robert Gordon University, Scotland, now permits the measurement of voltage changes around plants responding to gravity and light.

In addition to the unique technologies discussed above, the BRC aims to provide and, where possible, support conventional techniques for the study of ion transport. Equipment for single and double voltage and current clamp as well as patch clamp are now available. The Center also houses an Attolfluor ratio imager on generous long-term loan from Carl Zeiss Inc., continuing the close working relationship between this company, the Center, and the MBL.

Over the past year we hosted 35 visiting scientists investigating issues as diverse as the oxygen consumption of plants in space to several aspects of mammalian reproductive physiology and sterility. Our work focuses primarily on the transport of specific ions and their physiological function although some studies on the role of

generalized current patterning is still undertaken. Details of numerous collaborative ventures with outside visitors can be had by contacting the Resource Director. With in-house biomedical research we have been investigating calcium homeostasis in invertebrate neurons (PJSS) and anaerobic tolerance in vertebrate hepatocytes (SCL).

Staff

Smith, Peter J. S., Director and Associate Scientist
Hammar, Katherine, Research Assistant
Jaffe, Lionel F., Senior Scientist
Land, Stephen C., Postdoctoral Research Associate
McLaughlin, Jane A., Research Assistant
Sanger, Richard H., Senior Electronics Technician

Visiting Scientists

This past year the Research Center hosted 35 visitors, 26 from the United States with the remaining from Canada, France, Germany, Israel and the United Kingdom.

Boston University Marine Program

Faculty

Atema, Jelle, Professor of Biology, Director
Dionne, Vincent, Professor of Biology, Acting Director 1996-1997
Humes, Arthur G., Professor of Biology Emeritus
Kaufman, Les, Associate Professor of Biology
Lobel, Phillip, Associate Professor of Biology
Valiela, Ivan, Professor of Biology
Voigt, Rainer, Research Associate Professor
Ward, Nathalie, Lecturer

Staff

Burns, Jennifer, Course Coordinator
Corbett, Jean, Administrative Coordinator
Hahn, Dorothy, Senior Administrative Secretary
Hall, Sheri, Program Manager
Olson, Nancy, Executive Secretary
Pedersen, Jennifer, Program Assistant
Wheatley, Maryjo, Information and Development Officer

Postdoctoral Investigators

Basil, Jenny
Cebrian, Just
Cohen, Anne
Delay, Rona
Eisthen, Heather
Grasso, Frank
Trott, Tom

Visiting Faculty and Investigators

Epstein, Slava, Northeastern University
Hanlon, Roger, Marine Biological Laboratory
Hecker, Barbara, Rutgers University
Hinkle, Greg, Marine Biological Laboratory
Rommel, Sentiel, Smithsonian Institution
Simmons, William, Sandia National Laboratory
Solow, Andrew, Woods Hole Oceanographic Institution
Thayer, Charles, University of Pennsylvania
Wainwright, Norman, Marine Biological Laboratory



Graduate Students

PhD Students

Balint, Claire
 Batjakas, Ioannis
 Behr, Peter
 Dale, Jonathan
 Economakis, Alistair
 Farley, Lynda
 Hauxwell, Jennifer
 Herrold, Ruth
 Jefferson, Shawn
 Ma, Diana
 McClelland, James
 Oliver, Steven
 Ryan, Pamela
 Zhou, Qiao

MA Students

Ashcraft, Susan
 Baker, Colin
 Bell, Kimberly
 Demary, Kristian
 Ewell, Cara
 Filson, Jean
 Heiskell, Mary Beth
 Keith, Lucy
 Kerr, Lisa
 Paganessi, Laura
 Pitzer, Jocelyn
 Searcy, Brian
 Thompson, Sarah
 Timmer, Edward
 Valentini, Stephanie
 Wittenberg, Kim

Summer 1996 Interns

Carini, Stephen
 Chun, Nicol
 Dickens, Angela
 Distler, Matthew
 Dittmer, Kevin
 Feinstein, Noah
 Fritz, Cara
 Guenther, Carla
 Heckscher, Elizabeth
 Jimenez, Eris del C.
 LaBrecque, Erin
 Miller, Heather
 Nixon, Rebecca
 Sheikh, Sara
 Tober, Joanna
 Weston, Nathaniel
 Yelenik, Stephanie

Undergraduate Students

Bacabskas, Lisa
 Capra, Julie
 Chen, Linus
 Chi, Sam
 Frome, Ryan

Harris, Christopher
 Hurst, Veronica
 Jacukiewicz, Jennifer
 Jaffe, Adam
 Javonillo, Robert
 Kennedy, Bridget
 Kimble, Kathryn
 Kob, Makayla
 Lee, Kaitlin
 Macklin, Tim
 Mahar, Tara
 Margolis, Amy
 Matthew, Andrew
 Mehta, Jasmine
 Parikh, Bhavesh
 Pelletier, Derek
 Reddy, Chaitanya
 Rokeach, Brian
 Schlimmer, Lisa
 Shah, Anu

Laboratory of Jelle Atema

Many organisms use chemical signals as their main source of information about the environment. These signals are transported in the marine environment by turbulent currents, viscous flow, and molecular diffusion. Receptor organs extract signals through various physical and biological filtering processes. Currently, the lobster with its exquisite sense of taste and smell, is our major model to study the signal filtering capabilities of the whole animal and the tuning properties of its receptor cells. Research focuses on food signals and pheromones used in courtship and dominance, neurophysiology of receptor cells, behavior guided or modulated by chemical signals, computational models of odor plumes and neural filters, and underwater robotics. The work is fundamental for our understanding of the evolution of chemical sensing and signal processing in animals. Its applications are in aquatic resource management and in pollution monitoring.

Laboratory of Vincent Dionne

Odors are powerful stimuli. They can focus the attention, elicit behaviors (or misbehaviors), and even resurrect forgotten memories. These actions are directed by the central nervous system, but they depend upon the initial transduction of chemical signals by olfactory receptor neurons in the nasal passages. More than just a single process appears to underlie odor transduction, and the intracellular pathways that are used are far more diverse than once thought. Hundreds of putative odor receptor molecules have been identified that work through several different second messengers to modulate the activity of various types of membrane ion channels. Our studies are being conducted with aquatic salamanders using amino acids and other soluble chemical stimuli which these animals perceive as odors. Using electrophysiological and molecular approaches, the research examines how these cellular components produce odor detection, and how odors are identified and discriminated.

Laboratory of Arthur G. Humes

Research interests include systematics, development, host specificity, and geographical distribution of copepods associated with marine invertebrates. Current research is on taxonomic studies of copepods from invertebrates in the tropical Indo-Pacific area, and poecilostomatoid and siphonostomatoid copepods from deep-sea hydrothermal vents and cold seeps.

The Laboratory of Les Kaufman

Current research projects in the laboratory deal with speciation and extinction dynamics of haplochromine fishes in Lake Victoria. We are studying the systematics, evolution, and conservation genetics of a species flock encompassing approximately 700 very recently evolved taxa, in the dynamic and heavily impacted landscape of northern East Africa. In the lab, we are studying evolutionary morphology, behavior, and systematics of these small, brightly colored cichlid fishes. Another area of study is developmental and skeletal plasticity in fishes; we are studying the diversity of bone tissue types in fishes, differential response to mineral and mechanical challenge, and matrophic *versus* environmental effects in the development of coral reef fishes. We also study the biological basis for marine reserves in the New England fisheries. We are involved in collaborative research with NURC, NMFS, and others on the relative impact on groundfish stocks of juvenile habitat destruction *versus* fishing pressure.

Laboratory of Phillip Belol

Fishes are the most diverse vertebrate group and provide opportunities to study many aspects of behavior, ecology, and evolution. We primarily study how fish are adapted to different habitats and the behavioral ecology of species interactions. Current research focuses on fish acoustic communication. We are also conducting a long-term study of the marine biology of Johnston Atoll, Central Pacific Ocean. Johnston Atoll has been occupied continuously by the military since the 1930s and proved a unique opportunity for assessing the biological impacts of island industrialization and effects on reefs. Johnston Atoll is the site of the US Army's chemical weapons demilitarization facility, JACADS.

Laboratory of Ivan Valiela

Our major research activity involves the Waquoit Bay Land Margin Ecosystems Research Project (LMER). This work examines how human activity in coastal watersheds (including landscape use and urbanization) increases nutrient loading to groundwater and streams. Nutrients in groundwater are transported to the sea, and, after biogeochemical transformation, enter coastal waters. There, increased nutrients bring about a series of changes. The Waquoit Bay LMER is designed to help understand and model the coupling of land use and consequences to receiving waters, to study the processes involved, and to assess consequences and opportunities for coastal management.

A second long-term research topic is the structure and function of salt marsh ecosystems, including the processes of predation, herbivory, decomposition, and nutrient cycles.

Calcium Imaging Laboratory

This laboratory investigates the roles of calcium patterns in development. Our main tool uses the aequorins, a family of luminescent proteins ultimately obtained from a jellyfish and long studied by Dr. Osamu Shimomura at the MBL. Aequorins can either be microinjected into cells or transgenically expressed without disturbing function or development. The patterns of luminescence that are emitted by aequorinated cells reveals changing patterns and levels of free calcium with the cell or its progeny. Much of what we know about the role of calcium in development has been obtained with the aequorins.

The four systems under present or planned investigation are the *Drosophila* egg (in collaboration with Drs. Lynn Cooley and Carl Hashimoto at Yale), the zebrafish egg (in conjunction with Dr. Roger Hanlon at the MBL), the fucoid egg (in collaboration with Dr. Ken Robinson at Purdue), and the cellular slime mold, *Dictyostelium*.

Staff

Jaffe, Lionel, Senior Scientist
Créton, Robert, Research Associate
Steele, Margaret, Research Associate

Center for Advanced Studies in the Space Life Sciences at the MBL

(supported by the National Aeronautics and Space Administration)

The Marine Biological Laboratory and the National Aeronautics and Space Administration have established a cooperative arrangement with the formation of the Center for Advanced Studies in the Space Life Sciences at the MBL. This Center serves as an interface between NASA and the basic science community, addressing issues of mutual interest. A series of symposia, workshops, and seminars will be held at the MBL to advise NASA on a wide variety of topics in the life sciences, including cellular, molecular, developmental, plant, neuro-, and evolutionary biology. Special attention will be directed at examining how gravity and its control impact on biological processes, and how variations in gravity can be used as a probe to better understand such processes. This center will provide a forum for scientists to think and discuss, often for the first time, the role that gravity and other aspects of space flight may play in fundamental cellular and physiological processes. These interactions will also serve to inform the community of research opportunities in the life sciences that are of interest to NASA. In addition, a newsletter will be published to disseminate this information to a wider audience.

Staff

Dawidowicz, Lenny, Administrator
Nixon, Jennifer, Administrative Assistant

The Ecosystems Center

The Center carries out research and education in ecosystems ecology. Terrestrial and aquatic scientists work in a wide variety of ecosystems ranging from the streams, lakes and tundra of the Alaskan Arctic (limits on plant primary production) to sediments of Massachusetts Bay (controls of nitrogen cycling), to forests in New England (effects of soil warming on carbon and nitrogen cycling), and South America (effects on greenhouse gas fluxes of conversion of rain forest to pasture) and to large estuaries in the Gulf of Maine (effects of



plankton and benthos of nutrients and organic matter in stream runoff). Many projects, such as those dealing with carbon and nitrogen cycling in forests, streams, and estuaries, use the stable isotopes ^{13}C and ^{15}N to investigate natural processes. A mass spectrometer facility is available. Data from field and laboratory research are used to construct mathematical models of whole-system responses to change. Some of these models are combined with geographically referenced data to produce estimates of how environmental changes affect key ecosystem indexes such as net primary productivity and carbon storage throughout the world's terrestrial biosphere.

The results of the Center's research are applied, wherever possible, to the questions of the successful management of the natural resources of the earth. In addition, the ecological expertise of the staff is made available to public affairs groups and governmental agencies who deal with problems such as acid rain, coastal eutrophication, and possible carbon dioxide-caused climate change. Planning has begun for a Fall 1997 undergraduate semester course in environmental science. There are opportunities for postdoctoral fellows and graduate students.

Administrative Staff

Hobbie, John E., Co-Director
 Melillo, Jerry M., Co-Director
 Berthel, Dorothy J., Administrative Assistant
 Donovan, Suzanne J., Executive Assistant
 Foreman, Kenneth H., Associate Director of Environmental Studies Program
 Nuñez, Guillermo, Research Administrator
 Seifert, Mary Ann, Administrative Assistant
 Scanlon, Deborah G., Executive Assistant, LMER Coordination Office

Scientific Staff

Hobbie, John E., Senior Scientist
 Melillo, Jerry M., Senior Scientist
 Peterson, Bruce J., Senior Scientist
 Shaver, Gaius R., Senior Scientist
 Giblin, Anne E., Associate Scientist
 Hopkinson, Charles S., Associate Scientist
 Nadelhoffer, Knute J., Associate Scientist
 Deegan, Linda A., Associate Scientist
 Rastetter, Edward B., Associate Scientist
 Steudler, Paul A., Senior Research Specialist
 Neill, Christopher, Research Associate
 Pan, Yude, Research Associate
 Williams, Mathew, Research Associate
 Xiao, Xiangming, Research Associate

Educational Staff Appointments

Bret-Harte, M. Sydonia, Postdoctoral Research Associate
 Currie, William, Visiting Postdoctoral Scholar, U.S. Department of Agriculture
 Fernandes, David N., Postdoctoral Research Associate
 Gough, Laura, Postdoctoral Research Associate
 Herbert, Darrell A., Postdoctoral Research Associate
 Holmes, Robert M., Postdoctoral Research Associate
 Hughes, Jeffrey E., Postdoctoral Research Associate
 Stieglitz, Marc, NOAA Global Climate Change Postdoctoral Fellow
 Tian, Hanqin, Postdoctoral Research Associate
 Weaver, Melissa J., Postdoctoral Research Associate
 Vallino, Joseph J., Postdoctoral Research Associate

Technical Staff

Bahr, Michele P., Research Assistant
 Bettez, Neil D., Research Assistant
 Bryant, David M., Research Assistant
 Buffam, Ishi D., Research Assistant
 Canary, Jana D., Research Assistant
 Catricala, Christina E., Research Assistant
 Dornblaser, Mark M., Research Assistant
 Downs, Martha R., Research Assistant
 Garritt, Robert H., Senior Research Assistant
 Harvey, Christopher J., Research Assistant
 Helfrich, John V. K., III, Senior Research Assistant
 Kicklighter, David W., Senior Research Assistant
 Kwiatkowski, Bonnie L., Research Assistant
 Laundre, James A., Senior Research Assistant
 Micks, Patricia, Research Assistant
 Newkirk, Kathleen M., Research Assistant
 Nolin, Amy L., Research Assistant
 Regan, Kathleen M., Research Assistant
 Ricca, Andrea, Research Assistant
 Schwamb, Carol, Laboratory Assistant
 Slavik, Karie A., Research Assistant
 Tholke, Kristin S., Research Assistant
 Tucker, Jane, Senior Research Assistant
 Wollheim, Wilfred M., Research Assistant

Consultants

Bowles, Frances P., Research Systems Consultant Principal, Research Designs
 Bowles, Margaret C., Administrative Consultant
 Golden, Heidi E., Research Consultant

Visiting Scientists and Scholars

Banta, Gary, Visiting Scientist, Roskilde University
 Fry, Brian D., Visiting Scientist, Florida International University
 Gunderson, Per, Visiting Scientist, Danish Forest and Landscape Research Institute, Hørsholm, Denmark
 de Cássia Piccolo, Marisa, Visiting Scientist, CENA, University of Sao Paulo
 Seifert, Gabriel, CIEE Intern, Technical University of Wismar, Germany
 Zago, Christina, Visiting Scholar, Istituto per lo Studio della Dinamica delle Grandi Masse, ISDGM, Venice, Italy

Laboratory of Aquatic Animal Medicine and Pathology

The laboratory provides diagnostic, consultative research, and educational services to the institutions and scientists of the Woods Hole community concerned with marine animal health. Diseases of wild, captive, and cultured animals are investigated.

Staff

Aht, Donald A., Director and The Robert R. Marshak Term Professor of Aquatic Animal Medicine and Pathology, School of Veterinary Medicine, University of Pennsylvania
 Bullis, Robert A., Research Assistant Professor of Microbiology, University of Pennsylvania
 Leibovitz, Louis, Director Emeritus
 McCafferty, Michelle, Histology Technician, University of Pennsylvania

Moniz, Priscilla C., Administrative Assistant
 Smolowitz, Roxanna M., Senior Research Investigator in Pathology,
 University of Pennsylvania
 Wadman, Elizabeth A., Microbiology Technician, University of
 Pennsylvania

Laboratory of Aquatic Biomedicine

This laboratory investigates tumors of marine invertebrates by focusing on leukemias of soft shell clams. Monoclonal antibodies developed by this laboratory are being used to diagnose clam leukemia, identify and characterize a tumor-specific protein, and differentiate other leukemias in bivalve molluscs. The availability of molecular probes has enabled the laboratory to examine the expression of the p53 gene in both normal and leukemia cells. The impact of pollutants on clam leukemogenesis is currently being studied with an emphasis on regional superfund sites.

Staff

Reinisch, Carol L., Associate Scientist, MBL, and Chairperson,
 Department of Comparative Medicine, Tufts University School of
 Veterinary Medicine
 Barker, Colin, Research Assistant

Visiting Scientists

Brand, Dawna, Department of Environmental Studies, University of
 Victoria, Canada
 Powell, DeLois, Department of Biology, University of Maryland,
 Eastern Shore
 Strandberg, John, Department of Comparative Medicine, The Johns
 Hopkins School of Medicine

Students

Harper, Claudia, Tufts University School of Veterinary Medicine
 Smith, Cynthia, Tufts University School of Veterinary Medicine

Laboratory of Cell Communication

Established in 1994, this laboratory is devoted to the study of intercellular communication. The research focuses on the cell-to-cell channel, a membrane channel built into the junctions between cells. This channel provides one of the most basic forms of intercellular communication in organs and tissues. The work is aimed at the molecular physiology of this channel, in particular, at the mechanisms that regulate the communication. Electrophysiological-, fluorescent-tracer-, and molecular biological techniques are used to this end. As was recently discovered in this laboratory, the channel is the conduit of growth-regulating signals. It is instrumental in a basic feedback loop whereby cells in organs and tissues control their number; in a variety of cancer forms it is crippled. Work is aimed now at the mechanisms of growth control and at correcting cancer growth by transferring the gene for the cell-to-cell channel protein from normal cells into the cancer cells. Molecular genetic techniques are used in this endeavor.

Staff

Loewenstein, Werner, Senior Scientist
 Rose, Birgit, Senior Scientist
 Jillson, Tracy, Research Assistant

Laboratory of Barbara and Bruce Furie

γ -Carboxyglutamic acid is a calcium-binding amino acid that is found in the conopeptides of the predatory marine snail, *Conus*. This laboratory has been investigating the biosynthesis of this amino acid in *Conus* and the structural role of γ -carboxyglutamic acid in the conopeptides. Large numbers of *Conus* from Fiji, the Philippines, Hawaii, and Vietnam have been obtained and maintained in the Marine Resources Center.

Biosynthesis of γ -carboxyglutamic acid: The marine cone snail is the sole invertebrate known to synthesize γ -carboxyglutamic acid (Gla). The venomous cone snail produces neurotoxic conopeptides, some rich in Gla, which it injects into its prey. To examine the synthetic pathway for Gla, we have studied the *Conus* carboxylase. Of the *Conus* species tested, *C. bandanus*, *C. marmoreus*, *C. textile*, and *C. leopardus* had high specific γ -carboxylase activity. This activity has an absolute requirement for vitamin K. Western blot analysis using an anti-peptide antibody against bovine carboxylase revealed species at 55,000 and 110,000 molecular weight. Partially purified *Conus* carboxylase carboxylated proPT28 preferentially over FLEEL and a carboxy-conantokin G. These results suggest that *Conus* carboxylase substrates contain a carboxylation recognition site on a substrate precursor and that the mammalian and the *Conus* carboxylase share a common antigenic determinant. Furthermore, both enzymes exhibit both carboxylase and vitamin K epoxidase activity. The *Conus* vitamin K-dependent carboxylase should be an excellent model for determining the mechanism of action of vitamin K in the synthesis of γ -carboxyglutamic acid.

Gla-containing conopeptides: Six novel γ -carboxyglutamic acid-containing conopeptides have been isolated from the venom of *Conus textile*. The amino acid sequence, amino acid composition, and molecular weights of these peptides have been determined, but the biological activity remains unknown. Dr. Johan Stenflo, the scientist who discovered γ -carboxyglutamic acid in 1974, is the principal investigator of this project.

Structure of Gla-containing peptides: The three dimensional structure of conantokin G, a 17-residue conopeptide that contains 5 Gla residues, has been determined by 2-D ^1H -NMR spectra of conantokin G at pH 5.6 in the absence of calcium ions via identification of intrasidue spin systems using ^1H - ^1H through-bond connectivities. NOESY spectra provided d_{HN} , D_{NN} , and d_{HN} NOE connectivities and vicinal spin-spin coupling constants $^3J_{\text{HN}}$ were used to calculate F torsion angles. Structure generation used a set of 227 interproton distance restraints and a set of 13 torsion angle measurements to yield 20 convergent structures generated using distance geometry and simulated annealing methods. The backbone RMSD to the geometric average for 20 final structure is 0.76 Å. Conantokin G consists of a structured region from leucine 5 to arginine 13. This structure includes 1.6 turns of helix, which is preceded by a turn located between residues 4 and 8. The N-terminal region from residues 1 to 4, including Gla 3 and Gla 4, is unstructured. This is the first structure of a γ -carboxyglutamic acid-containing polypeptide that is not a member of the blood clotting family of proteins.

Staff

Furie, Barbara C., Scientist
 Furie, Bruce, Scientist
 Stenflo, Johan, Visiting Scientist
 Czerwec, Eva, Postdoctoral Fellow
 Rigby, Alan, Member of the Furie Laboratory in Boston

Laboratory of Roger Hanlon

This laboratory investigates the behavior and neurobiology of cephalopods. Studies of various learning capabilities are currently being conducted, as are studies on reproductive strategies that include agonistic behavior, female mate choice, and sperm competition. The latter studies involve DNA fingerprinting to determine paternity and help assess alternative mating tactics. Currently we are studying sensory mechanisms and function of polarization vision in cephalopods. Complimentary field studies are conducted locally and on coral reefs. The functional morphology and neurobiology of the chromatophore system of cephalopods are also studied on a variety of cephalopod species, and image analysis techniques are being developed to study crypsis and the mechanisms that enable cryptic body patterns to be neurally regulated by visual input.

Staff

Hanlon, Roger, Senior Scientist
Boal, Jean, Postdoctoral Fellow
Shashar, Nadav, Postdoctoral Fellow

Visiting Investigators

Gabr, Howaida, Graduate Student, Suez Canal University, Panama
Wittenberg, Kim, Graduate Student, Boston University Marine Program

Laboratory of Shinya Inoué

Scientists in this laboratory study the molecular mechanism and control of mitosis, cell division, cell motility, and cell morphogenesis, with emphasis on biophysical studies made directly on single living cells, especially developing eggs in marine invertebrates. Development of biophysical instrumentation and methodology, such as polarization optical and video microscopy and digital image processing techniques, and exploration of their underlying theory are an integral part of the laboratory's effort.

Staff

Inoué, Shinya, Distinguished Scientist
Knudson, Robert, Instrument Development Engineer
Leighton MacNeil, Jane, Executive Assistant
Maccaro, Jackie, Laboratory Assistant
Stemmer, Andreas, Visiting Assistant Scientist

Laboratory of Alan M. Kuzirian

Our research explores the functional morphology and ultrastructure of various organ systems in opisthobranch molluscs. The program includes mariculture of the nudibranch, *Hermisenda crassicornis*, with emphasis on developing reliable culture methods for rearing and maintaining the animal as a research resource. The process of metamorphic induction by natural and artificial inducers is being explored in an effort to understand the processes involved and as a means to increase the yield of cultured animals. Morphologic studies stress the ontogeny of neural and sensory structures associated with the photic and vestibular systems which have been utilized in learning and memory studies as well as the spatial and temporal occurrence of regulatory and transmitter neurochemicals. Concurrent with these morphologic studies is the development of new histologic techniques designed to facilitate the acquisition of morphologic and structural information supporting proposed physiologic processes.

Collaborative research includes histochemical investigations on strontium's role in initiating calcification in molluscan embryos (shell and statoliths), as well as immunocytochemical labeling of cell-surface antigens, secretory products, and intracellular organelles using mono- and polyclonal antibodies on squid giant axons and *Hermisenda* sensory and neurosecretory neurons, both *in situ* and in cell culture. Toxicity studies detailing the effects of lead on *Hermisenda* learning and memory, feeding, and the physiology of cultured neurons are also being conducted.

Additional collaborative research includes DNA fingerprinting using RAPD-PCR techniques in preparation for isogenic strain development of laboratory-reared *Hermisenda* and hatchery-produced bay scallops (*Argopectin irradians*) with distinct phenotypic markers for rapid field identification. The functional morphology and physiology of digestion in various marine metazoan invertebrates are being described. Systematic and taxonomic studies of nudibranch molluscs, to include molecular phylogenetics, are also of interest.

Staff

Kuzirian, Alan M., Associate Scientist
Bumann, Dirk, Postdoctoral Fellow

Visiting Scientists

Chikarmane, Hermant, Research Scientist, Aphios; Assistant Scientist, MBL
Clay, John R., National Institute for Neurological Disorders & Stroke, NIH
Leighton, Stephen B., Biomedical Engineering & Instrumentation Branch, NCRP-NIH

Laboratory of Rudolf Oldenbourg

The laboratory investigates the molecular architecture of living cells and of biological model systems using optical methods for imaging and manipulating these structures. For imaging non-invasively and non-destructively cell architecture dynamically and at high resolution, we have developed a new polarized light microscope (Pol-Scope). The Pol-Scope combines microscope optics with new electro-optical components, video, and digital image processing for fast analysis of specimen birefringence over the entire viewing field. Examples of biological systems currently investigated with the Pol-Scope are: microtubule-based structures (asters, mitotic spindles, single microtubules); actin-based structures (acrosomal process, stress fibers, nerve growth cones); zona pellucida of vertebrate oocytes; and biopolymer liquid crystals.

Staff

Oldenbourg, Rudolf, Associate Scientist
Katoh, Kaoru, Postdoctoral Research Associate
Knudson, Robert, Instrument Development Engineer
Maccaro, Jackie, Laboratory Assistant

Laboratory for Reproductive Medicine, Brown University and Woman and Infants Hospital, Providence

Work in this laboratory centers on the investigation of the underlying mechanisms behind female infertility. Particular emphasis is placed on the physiology of the oocyte or early embryo, with the aim of assessing developmental potential and mitochondrial dysfunction arising from mtDNA deletions. The studies taking place at the MBL

branch of the Brown Laboratory use some of the unique instrumentation available through the resident programs directed by Rudolf Oldenbourg and Peter J. S. Smith. Most particularly, non-invasive methods for oocyte and embryo study are being sought. Of several specific aims, one is to use the new Pol-Scope to analyze the birefringence of the preimplantation mammalian zona pellucida—a structure most predictive of successful implantation. We also hope to use this instrument to examine the mitotic spindles. An additional aim is to continue the studies on transmembrane ion transport using the non-invasive electro-physiological techniques available at the BioCurrents Research Center. Preliminary studies indicate that the calcium transport may form an accurate predictor of oocyte and embryo health. The newly developed oxygen probe also offers the possibility of looking directly at abnormalities in the mitochondria arising from accumulated mtDNA damage. Ultimately, in addition to investigating the mechanisms behind cellular aging underlying infertility, this laboratory aims to produce clinical methods for assessing preimplantation embryo viability, a development that will make a significant contribution to the health of women and children.

Staff

Keefe, David
Komminen, Kapura
Pepperell, John

Laboratory of Sensory Physiology

Members of this laboratory have conducted research on various facets of vision since 1973. Current investigations focus on vertebrate retinal photoreceptors. Light microscopy is used to study physical optical properties such as birefringence, dichroism, and visual pigment absorbance. The chemical basis of color vision is investigated principally with UV/VIS absorption spectroscopy. One aim is a thorough understanding of the chemistry that underlies spectral tuning. Other objectives are related to biophysical mechanisms that permit polarized light detection and wavelength-dependent discrimination in the ultraviolet and visible spectra.

Staff

Harosi, Ferenc I., Senior Scientist, MBL, and Boston University School of Medicine
Carleton, Karen L., Research Associate, University of New Hampshire
Kocher, Tomas D., Professor, University of New Hampshire
Navales, Inigo Flammarique, Graduate Student, University of Victoria
Van Keuren, Jeffrey R., Postdoctoral Student, University of Rhode Island

Laboratory of Osamu Shimomura

Biochemical mechanisms involved in the bioluminescence of various luminescent organisms are investigated. Based on the results obtained, various improved forms of bioluminescent and chemiluminescent probes are designed and produced for the measurements of intracellular free calcium and superoxide anion.

Staff

Shimomura, Osamu, Senior Scientist, MBL, and Boston University School of Medicine
Shimomura, Akemi, Research Assistant

Laboratory of Robert B. Silver

This laboratory studies how living cells make decisions. The focus of the research, typically using marine models, is on two main areas: the role of calcium in the regulation of mitotic cell division (sea urchins, sand dollars, etc.) and structure and function relationships of hair cell stereociliary movements in vestibular physiology (oyster, toadfish). Other related areas of study, *i.e.* synaptic transmission (squid), are also pursued. Tools include video light microscopy, multispectral, sub-wavelength, and very high speed (sub-millisecond frame rate) photon counting video light microscopy, telemanipulation of living cells and tissues, and modeling of decision processes. A cornerstone of the laboratory's analytical efforts is high performance computational processing and analysis of video light microscopy images. With luminescent, fluorescent, and absorptive probes, both empirical observation and computational modeling of cellular, biochemical, and biophysical processes permit interpretation and mapping of space-time patterns of intracellular chemical reactions and calcium signaling in living cells. A variety of *in vitro* biochemical, biophysical, and immunological methods are used. In addition to fundamental biological studies, the staff designs and fabricates optical hardware, and designs software for large video image data processing, analysis, and modeling.

Staff

Silver, Robert, Associate Scientist
Luders, Bruce, Research Assistant

Interns

LeDuc, Christine, REU Intern, Union College
Salomonte, Anita, REU Intern, University of Massachusetts, Amherst

Visiting Scientists

Att, Maxim, MikroPhotonische Universität von CZI, Germany
Kelley, Brian, Graduate Research Assistant, Cornell University
Prokop, Richard, Graduate Research Assistant, Cornell University
Reeves, Anthony, Cornell University
Stromboli, Emilio, Stazione de Napoli, Italy

The Marine Resources Center

The Marine Resources Center (MRC) is one of the world's most advanced facilities for maintaining and culturing aquatic organisms essential to advanced biological, biomedical, and ecological research. Service and education also play an important and complimentary role in the modern, 32,000-square-foot facility.

The MRC and its life support systems have already increased the ability of MBL scientists to conduct research and have inspired new concepts in scientific experiments. Vigorous research programs focusing on basic biological and biomedical aquatic models are currently being developed at the Center. These programs will enhance and build upon the MRC's existing research activities by the University of Pennsylvania's Laboratory of Aquatic Animal Medicine and Pathology (LAAMP) and in the Laboratory of Roger Hanlon.

In addition to research, the MRC provides a variety of services to the MBL community through its Aquatic Resources Division, the Water Quality and System Engineering Division, the Administrative Division, and the Laboratory for Aquatic Animal Medicine and Pathology.

Research and educational opportunities are available at the facility to established investigators, postdoctoral fellows, graduate, and undergraduate students. Investigators and students will find that the



MRC's unique life support and seawater engineering systems make this a favorable environment in which to conduct independent research and masters and doctoral theses using a variety of aquatic organisms and flexible tank space for customized experimentation on

live animals. Prospective investigators and students should contact the Director of the MRC for further information.

The MRC also hosts several courses: the annual AQUAVETS® courses sponsored by LAAMP, and an aquaculture course, the theme of which changes according to regional and national interests.

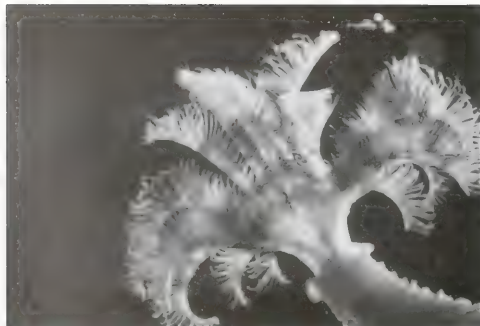
Staff

Hanlon, Roger, Director and Senior Scientist
Kuzirian, Alan, Associate Scientist
Boal, Jean, Postdoctoral Fellow
Shashar, Nadav, Postdoctoral Fellow

Visiting Investigators

Baker, Robert, New York University
Gilland, Edwin, Postdoctoral Fellow
Adamo, Shelly, Dalhousie University, Canada
Spotte, Stephen, University of Connecticut
Wittenberg, Kim, Graduate Student, Boston University Marine Program
Gabr, Howaida, Graduate Student, Suez Canal University, Panama

Honors



Friday Evening Lectures

June 21	James E. Rothman, Memorial Sloan-Kettering Cancer Center "Machinery and Mechanisms of Intracellular Transport and Synaptic Transmission"
June 28	Nicholas J. Strausfeld, University of Arizona "Evolution's masterpiece: insect brains and other antiquities (Don't swat me, I think)" (Lang Lecture)
July 5	Nancy Hopkins, Massachusetts Institute of Technology "Fishing for Developmentally Important Genes Using Zebrafish"
July 12	Doug Melton, Harvard University "Molecular Signal That Controls Vertebrate Embryogenesis"
July 18, 19	Masakazu Konishi, California Institute of Technology "How the Owl Tracks its Prey: Brain and Behavior" and "Recent Advances in Neurobiology of Birdsong" (Forbes Lectures)
July 26	Frank Press, Carnegie Institution of Washington "Out of Chaos—A Better Way to Support Science" (Lazarow/Stetten Lecture)
August 2	Susan Middleton and David Liittschwager, Endangered Species Program "Here Today, Gone Tomorrow? A Photographic Excursion Through the World of Endangered Species in North America"
August 9	Leroy Hood, Department of Molecular Biotechnology, University of Washington "Deciphering Human Heredity: A Revolution in Medicine of the 21st Century" (Glassman Lecture)
August 16	Gerald F. Joyce, Scripps Research Institute "The RNA World: Life Before DNA and Proteins"
August 23	Jared M. Diamond, Department of Physiology, University of California Medical Center, Los Angeles "Quantitative Evolutionary Design of Biological Systems"

Fellowships and Scholarships

In 1996, the MBL awarded research fellowships to 20 scientists from around the world. These fellows' research topics ranged from studying ion exchange and cardiovascular function using scanning confocal microscopy, to observing nerve activity and locomotion in crabs for improved designs in prosthesis and robots, to performing biochemical analysis of cell division cycles in surf clam eggs to advance understanding of uncontrolled cell multiplication in cancer. The MBL also awarded scholarships to 130 students in the MBL's summer courses.

The Science Writing Fellowships Program, now in its 11th year, brings print and broadcast journalists to the MBL each summer to participate in a hands-on laboratory course in cell and molecular biology followed by assignments in the courses and with independent investigators. Fellowships were awarded to 12 journalists in 1996.

In 1996, donors provided \$356,548 in support of the research fellowship program, \$85,000 for the Science Writing Fellowships Program, and \$207,889 to provide scholarships to students in MBL courses. Donors' names are noted below. Those individuals who received fellowships and scholarships follow.

Robert Day Allen Fellowship

Drs. Joseph and Jean Sanger

American Society for Cell Biology Scholarships

Dr. Elizabeth Marincola, Executive Director

MBL Associates Fellowships

MBL Associates

Frederik B. Bang Fellowship Fund

Mrs. Betsy G. Bang

Baxter Postdoctoral Fellowship Fund

Baxter International, Inc.

Frank A. Brown, Jr. Memorial Readership

Dr. Francis D. Carlson

The Jean and Katsuma Dan Fellowship Fund

Dr. John M. Arnold
Dr. Eloise E. Clark
Mr. And Mrs. Kenneth W. Cooper
Dr. and Mrs. David Epel
Dr. and Mrs. Teru Hayashi
Dr. and Mrs. Shinya Inoue
Mr. And Mrs. E. Dayton Jones
Klein and Melanie Pratt Merriman
Dr. Robert Bruce Nicklas
Drs. Joseph and Jean Sanger
Mrs. H. Burr Steinbach

Bernard Davis Fund

Dr. Porter W. Anderson, Jr.
Mrs. Elizabeth M. Davis

**Ann Osterhout Edison/
Theodore Miller Edison and
Olga Osterhout Sears/
Harold Bright Sears
Endowed Scholarship Fund**

Mr. Harold B. Sears

Daniel S. Grosch Scholarship Fund

Mrs. Edith T. Grosch

Aline D. Gross Scholarship Fund

Mrs. Mona Gross
Dr. Lewis P. Rowland
Mr. and Mrs. Alfred M. Weisberg

Ann E. Kammer Memorial Fellowship Fund

Dr. Ellen W. McLaughlin
Dr. Katherine A. Spielmann

Fred Karush Endowed Library Readership

Mrs. Fred Karush

William Randolph Hearst Educational Endowment

William Randolph Hearst Foundation

Merck Scholarship Fund

Merck & Co., Inc.

James A. and Faith Miller Fellowship Fund

Mrs. Charles Levie
Dr. and Mrs. David A. Miller

Mountain Memorial Fund

Mr. and Mrs. Dean C. Allard, Jr.
Ms. Brenda J. Bodian
Ms. Elinor W. Bodian
Ms. Helen Bodian & Mr. Roger Alcaly
Mrs. Mary S. Brooks
Dr. and Mrs. John B. Buck
Mr. and Mrs. Ian D. W. Cramer
Mrs. James R. Glazebrook
Ms. Elizabeth J. Goulett
Col. And Mrs. William E. John
Dr. Katherine Keenan
Ms. Anne C. Kimball
Mrs. Edward King
Mr. Keith G. Kozminski
Mr. and Mrs. John H. Langdon
Mr. and Mrs. Harry Mellins
Mrs. Mary E. Montgomery
Dr. Julia S. Rankin
Readers' Digest Foundation
Mrs. Barbara M. Roberts
Mr. and Mrs. Thomas H. Roberts
Dr. Joel L. Rosenbaum
Mr. and Mrs. William B. Sanford
Mr. and Mrs. Hans L. Schlesinger
Dr. and Mrs. R. Walter Schlesinger
Joel W. Silverstein, M.D.
Mr. Heinz Specht
Mr. and Mrs. Richard C. Steere
Mr. and Mrs. John W. Stewart
Mrs. Ann S. Youmans

Nikon Fellowship

Nikon, Inc.

Philip H. Presley Memorial Scholarships

Carl Zeiss, Inc.

Howard A. Schneiderman Endowed Scholarship

Mrs. Howard Schneiderman

Science Writing Fellowships Program

American Society for Biochemistry and Molecular Biology

American Society for Cell Biology
American Society for Microbiology
American Society for Neurochemistry
Association for Research in Vision and Ophthalmology
Biophysical Society
Charles A. Dana Foundation
FASEB
Foundation for Microbiology
Friendship Fund
John S. and James L. Knight Foundation
New York Times Company Foundation
The Ottaway Foundation
Society for Integrative and Comparative Biology
The Washington Post Company

The Moshe Shilo Memorial Scholarship Fund

Dr. Seymour S. Cohen
Dr. Arnold Demain
Dr. Edward Goldberg
Sir Hans Kornberg
Dr. J. Gijis Kuenen
Dr. John J. Lee
Dr. Laszlo Lorand
Dr. Richard I. Mateles

The Evelyn and Melvin Spiegel Fellowship Fund

Drs. Joseph and Jean Sanger
The Sprague Foundation

H. B. Steinbach Fellowship

Mrs. H. Burr Steinbach

The Walter L. Wilson Endowed Scholarship Fund

Mrs. Irmgard Alexander
Paul N. Chervin, M.D.
Mrs. Marian Rigauumont
Dr. Jean R. Wilson

Young Scholars/Fellows Program

Mr. and Mrs. William M. Ferry
Dr. and Mrs. Prosser Gifford
Dr. Birgit Rose Loewenstein
Dr. Charles Lowe
Dr. and Mrs. William M. McDermott
Dr. Arthur B. Pardee
Dr. and Mrs. Philip Person
Mrs. Atholie K. Rosett
Dr. and Mrs. Thomas R. Stetson

Post-Course Research Support Provided by:

Theodore D. Inoue
Carl Zeiss, Inc.

Fellowships Awarded

MBL Summer Research Fellows

• Luis Beaugé, supported by the *MBL Associates, Esther A. and Joseph Klingenstein, MBL Research, and Frank R. Lillie Fellowship Funds*, is an M.D., PhD at the Instituto M. & M. Ferreyra in Argentina. His research project was "Na/Ca exchange across the plasma membrane of squid axons: Mechanisms of metabolic control of the Na/Ca exchanger."

• Jim H. Belanger, an *MBL Associates Fellow*, is a research associate in the Division of Neurobiology at the University of Arizona. His research project was "Gain-setting as a tactic for load compensation in crustacean neuromuscular control."

• Joshua R. Berlin, supported by the *MBL Research and the Horace W. Stunkard Fellowship Funds*, is a research scientist at the Bockus Research Institute in Philadelphia, Pennsylvania. His research project was "Regulation of unitary SR calcium release events in muscle."

• Jean Geary Boal, supported by the *Ann E. Kammer and MBL Research Fellowship Funds*, is a post-doctoral fellow at the University of Texas Marine Biomedical Institute. Her research project was "Spatial learning: Brain size or life history demands?"

• Wei-Jun Cai, a *Bernard Davis Fellow*, is Assistant Professor in the Department of Marine Sciences at the University of Georgia. Dr. Cai's project was "Microelectrode study of carbon cycling in marine and estuarine sediments."

• Paul A. Garriss, supported by the *Stephen W. Kuffler and H. Burr Steinbach Fellowship Funds*, is Assistant Professor in the Department of Biological Sciences at Illinois State University. His research project was "Real-time measurement of biogenic amines in the lobster."

• Robert M. Gould, an *Esther A. and Joseph Klingenstein Fellow*, is a Research Scientist at the New York State Institute for Basic Research, Staten Island. His research project was "Differences in rat chondrichthys oligodendrocytes."

• Avram Hershko, the *Herbert W. Rand Fellow*, is Professor of Biochemistry at Technion-Israel Institute of Technology, in Haifa. His research project was "Mechanisms of cell-cycle regulated cyclin degradation."

• David B. Jaffe, supported by the *Charles R. Crane, John O. Crane, and M. G. F. Fuortes Fellowship Funds*, is Assistant Professor in the Division of Life Sciences at the University of Texas, San Antonio. His research project was "Imaging calcium dynamics in hippocampal CA3 pyramidal neurons."

• Robert J. Kunz, supported by the *Frank A. Brown, Jr. Readership and MBL Associates Fellowship Funds*, is Executive Editor at *Discover* magazine. Mr. Kunz spent his summer completing *To the Sea*—his book about marine science.

• Jeff W. Lichtman, a *Nikon Fellow*, is Professor of Neurobiology in the Department of Anatomy at the Washington University School of Medicine in St. Louis, Missouri. His research project was "Reflected light confocal imaging of the postsynaptic membrane."

• Mark Q. Martindale, supported by the *Bernard Davis and the Evelyn and Melvin Spiegel Fellowship Funds*, is Assistant Professor in the Department of Organismal Biology at the University of Chicago, Illinois. His research project was "Development and regeneration in ctenophores."

• Timothy S. McClintock, a *Lucy B. Lemann Fellow*, is Assistant Professor in the Department of Physiology at the University

of Kentucky. His research project was "Molecular mechanisms of signal integration in lobster olfactory receptor neurons."

• Fumio Oosawa, a *Frank R. Lillie Fellow*, is in the Department of General Education at Aichi Institute of Technology in Toyota, Japan. His research focused on the motor proteins myosin, actin and kinesin which are integral to intracellular organelle movement and synaptic transmission.

• Haohua Qian, the *Erik B. Fries Fellow*, is a post-doctoral fellow at the Biological Laboratories at Harvard University in Cambridge, Massachusetts. Dr. Qian's research project was "Modulation of GABA_A receptors in skate retina."

• Johan Stenflo, a *Herbert Rand Fellow*, a visiting scientist from the University of Lund, Sweden, worked with Drs. Bruce and Barbara Furie on studies of the neuropeptides synthesized by cone snails which are important to the analysis of neuroreceptors and ion channels.

• Joel Tabb, supported by the *Robert Day Allen and MBL Associates Fellowship Funds*, is a research associate in the Department of Biology at Dartmouth College in Hanover, New Hampshire. His research project was "Motility of endoplasmic reticulum in squid axon."

• Judith M. Venuti, supported by the *Frederik B. Bang and MBL Associates Fellowship Funds*, is Assistant Professor in the Department of Anatomy and Cell Biology at Columbia University in New York. Her research project was "The role of extracellular signaling in myogenic specification."

• James Walker, supported by the *James A. and Faith Miller Memorial and the MBL Associates Fellowship Funds*, is a graduate student in the Department of Biochemistry at the University of Cambridge, UK. His research project was "The TPR protein in clam oocytes."

• Samuel Ward, a *Bernard D. Davis Fellow*, is Professor in the Department of Molecular and Cellular Biology at the University of Arizona in Tucson. His research project was "The evolution of crawling sperm."

Grass Fellows

• Jennifer A. Basil, Ph.D., Boston University Marine Program. Project title: *Nautilus* chemosensory orientation behavior.

• Mary E. T. Boyle, Ph.D., La Jolla Cancer Research Center. Project title: The role of contactin /F11 in chick retinal synaptogenesis.

• Melissa J. Coleman, Ph.D., Brandeis University. Project title: Functional role of CaM kinase in synaptic plasticity.

• Sabine Hilfiker-Rothenfluh, The Rockefeller University. Project title: The role of rabphilin-3A phosphorylation in neurotransmitter release at the giant synapse of the squid, *Loligo pealei*.

• Shyue-fang Hsu, Ph.D., University of Wisconsin-Madison Medical School. Project title: Kinetic studies of calcium-triggered secretion from nerve terminals of squids.

• Alexey Melishechuk, Ph.D., University of Pennsylvania. Project title: Localization of amino acids forming the activation gate of the potassium channel.

- Teresa Nick, Yale University. Project title: Comparison of Ca^{++} currents in growth cones and somata of frog sympathetic neurons.
- Orian Shirihai, The Technion, Israel. Project title: Role of potassium inward rectifier to phagocytosis.
- Graciela Unguez Munoz, Ph.D., University of Texas. Project title: Development of electrolytes: electrophysiological and molecular studies.
- Claudia Wiedemann, Friedrich-Miescher Institute. Project title: Importance of phospholipidylasitides for cortical granule secretion in sea urchin eggs.
- Jian Zhang, SUNY, Buffalo. Project title: The functional studies of GABA receptors expressed in skate ganglion cells.

MBL Science Writing Fellows

Leigh Fenly, *San Diego Union-Tribune*
 Karen Fox, *Science Report* (radio)
 Josie Glausiusz, *Discover* magazine
 Kevin Krajick, *Newsweek*
 Richard Lipkin, Freelance, New York, NY
 Kathleen McAuliffe, Freelance, Coral Gables, FL
 Ridgely Ochs, *Newsday*
 Wallace Raven, Freelance, Oakland, CA
 Kathy Sawyer, *The Washington Post*
 Julie Titone, *The Spokesman-Review*
 Lisa Seachrist, Freelance, Bethesda, MD
 Mark Ward, *Milwaukee Journal Sentinel*

SPINES—Summer Program in Neuroscience Ethics and Survival

SPINES is a month-long program directed by Joe L. Martinez, Jr., and James Townsel. The program is supported by grants from NIMH administered by the American Psychological Association and the Association of Neuroscience Departments and Programs. SPINES offers an introduction to the opportunities available at the MBL and in the field of neuroscience in general. Fellows are taught responsible conduct in research and other survival skills such as scientific writing, poster construction, presentations, grant mechanisms, and how to seek a postdoctoral or job position.

Predoctoral
 Marcides Acosta-Martinez
 Walter Almenza
 Terrence Brannon
 Deanna Brown
 Raymond Chitwood
 Dianne Cruz
 Veronica Galvan
 Carlos Jaramillo
 Carlos Mejias Aponte
 Pablo Monsivias
 Christov Roberson
 Alba Rodriguez
 Ansalan Stewart
 Sherri Stewart
 Alejandro Terrazas
 Robin Wellington
 Nicole Wicha

Postdoctoral
 Sharon Perez

Scholarships Awarded

American Society for Cell Biology Scholarships

Pedro Santiago, University of Puerto Rico
 Sonya Summerour, University of California, San Diego
 Paul Gray, University of California
 Joseph Sisneros, Florida Institute of Technology
 Sheryl Starsinic, University of California, Davis
 Jose Perez-Jimenez, University of Puerto Rico
 Jeffrey Milner, University of Alabama
 Pablo Monsivais, University of Washington
 Rachel Fernandes, University of Connecticut

American Psychological Association

Jonathan Pierce, University of Oregon
 Pablo Monsivais, University of Washington

Biology Club of the City of New York Scholarships Fund

Wei Weng, Columbia University

C. Lalor Burdick Fellowship Fund

Matthew D. Clark, Max-Planck Institute, Berlin, Germany

The Burroughs Wellcome Fund

Marion Doerner, University of Munich, Germany
 Andrea Graham, Cornell University
 Loic Dupré, Institut Pasteur de Lillie, France
 Rhian Hayward, University of Oxford, UK
 Karl Hoffmann, Johns Hopkins University
 Stenio Fragos, Oswaldo Cruz Foundation
 Kirkwood Land, University of California
 Julie Gauthier, University of Maryland
 Yasuhiro Morita, Johns Hopkins University
 Edward Ngau, University of Nairobi, Kenya
 Harris Rotman, Thomas Jefferson University
 Stepanka Vanacova, Charles University, Czechoslovakia
 Lita Vieira, Hebrew University of Jerusalem, Israel

Gary Nathan Calkins Memorial Scholarship Fund

James D. Jontes, Scripps Research Institute
 John S. Mitcheson, University of Bristol, England, UK
 Jorge S. Olmos, Universidad Nacional Autonoma De Mexico, Cuernavaca, Mexico

Edwin Grant Conklin Scholarship Fund

Michal Shteinberg-Blum, Israel Institute Technology, Israel
Adele Wright, Georgia Institute of Technology

Bernard Davis Fund

Ana Fernandez, Facultad de Quimica, Uruguay
Daniel Gisi, Swiss Fed Institute of Technology
Stefan Ratering, Max-Planck-Institut, Göttingen, Germany
Annick Wilmotte, Université de l'Etat à Liège, France

William F. and Irene C. Diller Scholarship Fund

Ilya Shekhter, Boston University
Andrew Swensen, Brandeis University

Caswell Grave Scholarship Fund

Srdjan D. Antic, Institute for Biological Research, Belgrade, Yugoslavia
Edwin E. Gilliam, University of Arizona
Celine G. Auger, Max-Planck-Institut, Göttingen, Germany
Bruce E. Cohen, University of California, Berkeley
Kristina D. Micheva, University of Montreal, Canada
Shaowen Bao, University of Southern California
Daniel T. Stimson, University of Arizona
Birke Bartosch, Clare Hall Laboratories, South Mimms, England
James D. Jontes, Scripps Research Institute
Leana M. Topper, University of Virginia, Charlottesville

Daniel S. Grosch Endowed Scholarship Fund

Mark O. Martin, Occidental College, Los Angeles
Jose R. Perez-Jimenes, University of Puerto Rico, Mayaguez

Aline D. Gross Scholarship Fund

Shari E. Gelber, Columbia University, New York, NY
Caitlin M. Smith, Yale University
Ana Fernandez Scavino, Facultad De Quimica, Montevideo, Uruguay

William Randolph Hearst Educational Endowment Scholarships

Edwin Gilliam, University of Arizona

Howard Hughes Medical Institute Educational Program Scholarship Funding

Vishwas R. Agashe, National Center for Biological Science, India
Magdalena Chrzanoska-Wo, University of North Carolina
Birke Bartosch, Clare Hall Laboratories, South Mimms, England, UK
Bryan H. Clubb, University of Western Ontario, Canada
Matteo Caleo, Istituto Neurofisiologia CNR, Pisa, Italy
Oxana Karpenki, Brown University
John S. Mitcheson, University of Bristol, England, UK
Michael Shteinberg-Blum, Israel Institute Technology
Arpita Upadhyaya, University of Notre Dame
Kristina D. Micheva, University of Montreal, Canada
Celine G. Auger, Max-Planck-Institut, Göttingen, Germany
Venugopala Reddy, Tata Institute of Fundamental Research, India

Arthur Klorfein Scholarship Fund

Bryan H. Clubb, University of Western Ontario, Canada
Jennifer M. King, Duke University, Durham, North Carolina
Elizabeth Stillwell, Emory University
Amber Wells, University of Pennsylvania
Sacha Glardon, University of Basel, Switzerland
Darren Williams, Southampton University, England, UK
William Metcalf, University of Illinois
Volker Bruchert, Indiana University, Bloomington

Frank R. Lillie Scholarship Fund

Vishwas R. Agashe, University of Bayreuth, Bayreuth, Germany
Venugopala Reddy, Tata Institute of Fundamental Research, India
Tamira Elul, University of California, Berkeley
Guojun Sheng, Rockefeller University
Youn-Ho Lee, California Institute of Technology
Brian Harfe, Johns Hopkins University
Michal Shteinberg-Blum, Israel Institute Technology, Israel

Jacques Loeb Founders' Scholarship Fund

Sophie R. M. Janssens, Janssen Research Foundation, Beerse, Belgium

MBL Pioneer Endowment

Srdjan Antic, Institute for Biological Research, Yugoslavia
Birgit Ehmer, University of Würzburg, Germany
Harm Vitzthum, University of Regensburg, Germany
Ana Fernandez Scavino, Facultad de Quimica, Montevideo, Uruguay

S. O. Mast Founders' Scholarship Fund

Vishwas R. Agashe, University of Bayreuth, Bayreuth, Germany
Matteo Caleo, Istituto Neurofisiologia CNR, Pisa, Italy
Jesse G. Dillon, University of Oregon, Eugene

Mountain Memorial Fund

Vivek C. Abraham, Carnegie Mellon University
Matteo Caleo, Istituto Neurofisiologia CNR, Pisa, Italy
Bryan H. Clubb, University of Western Ontario, Canada
James D. Jontes, Scripps Research Institute
Jennifer M. King, Duke University
Leana M. Topper, University of Virginia

Planetary Biology Institute Scholarships

Rachel Greedy, University of Leicester, England, UK
Jay Gulledge, University of Alaska

William Townsend Porter Scholarship Fund

Pedro Santiago, University of Puerto Rico
Sonya Summerour, University of California, San Diego
Paul Gray, University of California
Joseph Sisneros, Florida Institute of Technology
Jose Perez-Jimenez, University of Puerto Rico
Jeffrey Milner, University of Alabama

Phillip H. Presley Memorial Scholarships

Veronica Maubecin Alvarez, University of Buenos Aires, Argentina
 Hinrich Böger, Max-Planck-Institut, Germany
 Helen Faulkner, University of Manchester, England, UK
 Anna Laura Salvati, Università di Palermo, Italy

Herbert W. Rand Scholarship Fund

Birke Bartosch, Clare Hall Laboratories, South Mimms, England, UK
 Johanna Klerkz, Utrecht University, The Netherlands
 Rie Kusakaabe, Kyoto University, Japan
 Wei Weng, Columbia University
 Darren Williams, Southampton University, England, UK
 Yingzi Xue, Columbia University

Society for Developmental Biology Scholarships

Anna Edlund, University of Virginia
 Robert Peterson, University of Florida
 Michael Shapiro, Harvard University
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Zukin, R. Suzanne, Albert Einstein College of Medicine, Dept. of Neuroscience, 1410 Pelham Parkway South, Bronx, NY 10461

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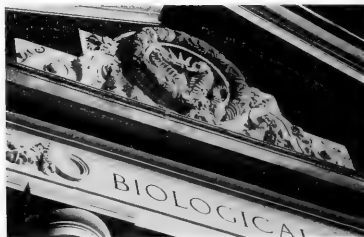
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Certificate of Organization Articles of Amendment Bylaws



Certificate of Organization

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is
THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirtieth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

Charles Endicott
Commissioner of Corporations)

Articles of Amendment

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

Voted: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

"No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.

"Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

"The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of \$10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

Paul Guzzi
Secretary of the Commonwealth)

Bylaws

(Revised August 7, 1992 and December 10, 1992)

ARTICLE I—THE CORPORATION

A. *Name and Purpose.* The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and main-

tain a laboratory or station for scientific study and investigation and a school for instruction in biology and natural history.

B. Nondiscrimination. The Corporation shall not discriminate on the basis of age, religion, color, race, national or ethnic origin, sex or sexual preference in its policies on employment and administration or in its educational and other programs.

ARTICLE II—MEMBERSHIP

A. Members. The Members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees (the "Board"), upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by the Board. At any regular or special meeting of the Board, the Board may elect new Members. Members shall have no voting or other rights with respect to the Corporation or its activities except as specified in these Bylaws, and any Member may vote at any meeting of the Members in person only and not by proxy. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has retired from his or her home institution may, upon written request to the Corporation, be designated a Life Member. Life Members shall not have the right to vote and shall not be assessed for dues.

B. Meetings. The annual meeting of the Members shall be held on the Friday following the first Tuesday in August of each year, at the Laboratory of the Corporation in Woods Hole, Massachusetts, at 9:30 a.m. The Chairperson of the Board shall preside at meetings of the Corporation. If no annual meeting is held in accordance with the foregoing provision, a special meeting may be held in lieu thereof with the same effect as the annual meeting, and in such case all references in these Bylaws, except in this Article II.B., to the annual meeting of the Members shall be deemed to refer to such special meeting. Members shall transact business as may properly come before the meeting. Special meetings of the Members may be called by the Chairperson or the Trustees, and shall be called by the Clerk, or in the case of the death, absence, incapacity or refusal by the Clerk, by any other officer, upon written application of Members representing at least ten percent of the smallest quorum of Members required for a vote upon any matter at the annual meeting of the Members, to be held at such time and place as may be designated.

C. Quorum. One hundred (100) Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person at a meeting attended by a quorum shall constitute action on behalf of the Members.

D. Notice of Meetings. Notice of any annual meeting or special meeting of Members, if necessary, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting at least 15 days before such meeting to each Member at his or her address as shown on the records of the Corporation.

E. Waiver of Notice. Whenever notice of a meeting is required to be given a Member, under any provision of the Articles or Organization or Bylaws of the Corporation, a written waiver thereof, executed before or after the Meeting by such Member, or his or her duly authorized attorney, shall be deemed equivalent to such notice.

F. Adjournments. Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present at the meeting, whether or not such Members constitute a quorum, or by any officer entitled to preside at or to act as Clerk of such meeting, if no Member is present or represented. It shall not be necessary to notify any Members of any adjournment unless no Member is present or represented at the meeting which is adjourned, in which case, notice of the adjournment shall be given in accordance with Article II.D. Any business which could have been transacted at any meeting of the Members as originally called may be transacted at an adjournment thereof.

ARTICLE III—ASSOCIATES OF THE CORPORATION

Associates of the Corporation. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees. The Associates of the Marine Biological Laboratory shall have no voting rights.

ARTICLE IV—BOARD OF TRUSTEES

A. Powers. The Board of Trustees shall have the control and management of the affairs of the Corporation. The Trustees shall elect a Chairperson of the Board who shall serve until his or her successor is elected and qualified. They shall annually elect a President of the Corporation. They shall annually elect a Vice

Chairperson of the Board who shall be Vice Chairperson of the meetings of the Corporation. They shall annually elect a Treasurer. They shall annually elect a Clerk, who shall be a resident of Massachusetts. They shall elect Trustees-at-Large as specified in this Article IV. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They shall choose such other officers and agents as they shall think best. They may fix the compensation of all officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board shall have the power to choose an Executive Committee from their own number as provided in Article V, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article V. They shall, from time to time, elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

B. Composition and Election.

(1) The Board shall include 24 Trustees elected by the Board as provided below:

(a) At least six Trustees ("Corporate Trustees") shall be Members who are scientists, and the other Trustees ("Trustees-at-Large") shall be individuals who need not be Members or otherwise affiliated with the Corporation.

(b) The 24 elected Trustees shall be divided into four classes of six Trustees each, with one class to be elected each year to serve for a term of four years, and with each such class to include at least one Corporate Trustee. Such classes of Trustees shall be designated by the year of expiration of their respective terms.

(2) The Board shall also include the Chief Executive Officer, Treasurer and the Chairperson of the Science Council, who shall be *ex officio* voting members of the Board.

(3) Although Members or Trustees may recommend individuals for nomination as Trustees, nominations for Trustee elections shall be made by the Nominating Committee in its sole discretion. The Board may also elect Trustees who have not been nominated by the Nominating Committee.

C. Eligibility. A Corporate Trustee or a Trustee-at-Large who has been elected to an initial four-year term or remaining portion thereof, of which he/she has served at least two years, shall be eligible for re-election to a second four-year term, but shall be ineligible for re-election to any subsequent term until one year has elapsed after he/she has last served as a Trustee.

D. Removal. Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or, for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee to be so removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him or her.

E. Vacancies. Any vacancy in the Board may be filled by vote of a majority of the remaining Trustees present at a meeting of Trustees at which a quorum is present. Any vacancy in the Board resulting from the resignation or removal of a Corporate Trustee shall be filled by a Member who is a scientist.

F. Meetings. Meetings of the Board shall be held from time to time, not less frequently than twice annually, as determined by the Board. Special meetings of Trustees may be called by the Chairperson, or by any seven Trustees, to be held at such time and place as may be designated. The Chairperson of the Board, when present, shall preside over all meetings of the Trustees. Written notice shall be sent to a Trustee's usual or last known place of residence at least two weeks before the meeting. Notice of a meeting need not be given to any Trustee if a written waiver of notice executed by such Trustee before or after the meeting is filed with the records of the meeting, or if such Trustee shall attend the meeting without protesting prior thereto or at its commencement the lack of notice given to him or her.

G. Quorum and Action by Trustees. A majority of all Trustees then in office shall constitute a quorum. Any meeting of Trustees may be adjourned by vote of a majority of Trustees present, whether or not a quorum is present, and the meeting may be held as adjourned without further notice. When a quorum is present at any meeting of the Trustees, a majority of the Trustees present and voting (excluding abstentions) shall decide any question, including the election of officers, unless otherwise required by law, the Articles of Organization or these Bylaws.

H. Transfers of Interests in Land. There shall be no transfer of title nor long-term lease of real property held by the Corporation without prior approval of not less than two-thirds of the Trustees. Such real property transactions shall be finally acted upon at a meeting of the Board only if presented and discussed at a prior

meeting of the Board. Either meeting may be a special meeting and no less than four weeks shall elapse between the two meetings. Any property acquired by the Corporation after December 1, 1989 may be sold, any mortgage or pledge of real property (regardless of when acquired) to secure borrowings by the Corporation may be granted, and any transfer of title or interest in real property pursuant to the foreclosure or endorsement of any such mortgage or pledge of real property may be effected by any holder of a mortgage or pledge of real property of the Corporation, with the prior approval of not less than two-thirds of the Trustees (other than any Trustee or Trustees with a direct or indirect financial interest in the transaction being considered for approval) who are present at a regular or special meeting of the Board at which there is a quorum.

ARTICLE V—COMMITTEES

A. *Executive Committee.* There shall be an Executive Committee of the Board of Trustees which shall consist of not more than eleven (11) Trustees, including *ex officio* Trustees, elected by the Board.

The Chairperson of the Board shall act as Chairperson of the Executive Committee and the Vice Chairperson as Vice Chairperson. The Executive Committee shall meet at such times and places and upon such notice and appoint such subcommittees as the Committee shall determine.

The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board except those powers specifically withheld, from time to time, by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may, from time to time, approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board.

The Executive Committee shall keep appropriate minutes of its meetings, which shall be reported to the Board. Any actions taken by the Executive Committee shall also be reported to the Board.

B. *Nominating Committee.* There shall be a Nominating Committee which shall consist of not fewer than four nor more than six Trustees appointed by the Board in a manner which shall reflect the balance between Corporate Trustees and Trustees-at-Large on the Board. The Nominating Committee shall nominate persons for election as Corporate Trustees and Trustees-at-Large, Chairperson of the Board, Vice Chairperson of the Board, President, Treasurer, Clerk, Director of the Laboratory and such other officers, if any, as needed, in accordance with the requirements of these Bylaws. The Nominating Committee shall also be responsible for overseeing the training of new Trustees. The Chairperson of the Board of Trustees shall appoint the Chairperson of the Nominating Committee. The Chairperson of the Science Council shall be an *ex officio* voting member of the Nominating Committee.

C. *Science Council.* There shall be a Science Council (the "Council") which shall consist of Members of the Corporation elected to the Council by vote of the Members of the Corporation, and which shall advise the Board with respect to matters concerning the Corporation's mission, its scientific and instructional endeavors, and the appointment and promotions of persons or committees with responsibility for matters requiring scientific expertise. Unless otherwise approved by a majority of the members of the Council, the Chairperson of the Council shall be elected annually by the Council. The chief executive officer of the Corporation shall be an *ex officio* voting member of the Council.

D. *Board of Overseers.* There shall be a Board of Overseers which shall consist of not fewer than five nor more than eight scientists who have expertise concerning matters with which the Corporation is involved. Members of the Board of Overseers may or may not be Members of the Corporation and may be appointed by the Board of Trustees on the basis of recommendations submitted from scientists and scientific organizations or societies. The Board of Overseers shall be available to review and offer recommendations to the officers, Trustees and Science Council regarding scientific activities conducted or proposed by the Corporation and shall meet from time to time, not less frequently than annually, as determined by the Board of Trustees.

E. *Board Committees Generally.* The Trustees may elect or appoint one or more other committees (including, but not limited to, an Investment Committee, a Development Committee, an Audit Committee, a Facilities and Capital Equipment Committee and a Long-Range Planning Committee) and may delegate to any such committee or committees any or all of their powers, except those which by law, the Articles of Organization or these Bylaws the Trustees are prohibited from delegating; provided that any committee to which the powers of the Trustees are delegated shall consist solely of Trustees. The members of any such committee shall have such tenure and duties as the Trustees shall determine. The Investment Committee, which shall oversee the management of the Corporation's endow-

ment funds and marketable securities shall include as *ex officio* members, the Chairperson of the Board, the Treasurer and the Chairperson of the Audit Committee, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business, but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

F. *Actions Without a Meeting.* Any action required or permitted to be taken at any meeting of the Executive Committee or any other committee elected by the Trustees may be taken without a meeting if all members of such committees consent to the action in writing and such written consents are filed with the records of meetings. Members of the Executive Committee or any other committee elected by the Trustees may also participate in any meeting by means of a telephone conference call, or otherwise take action in such a manner as may, from time to time, be permitted by law.

G. *Manual of Procedures.* The Board of Trustees, on the recommendation of the Executive Committee, shall establish guidelines and modifications thereof to be recorded in a Manual of Procedures. Guidelines shall establish procedures for: (1) Nomination and election of members of the Corporation, Board of Trustees and Executive Committee; (2) Election of Officers; (3) Formation and Function of Standing Committees.

ARTICLE VI—OFFICERS

A. *Enumeration.* The officers of the Corporation shall consist of a President, a Treasurer and a Clerk, and such other officers having the powers of President, Treasurer and Clerk as the Board may determine, and a Director of the Laboratory. The Corporation may have such other officers and assistant officers as the Board may determine, including (without limitation) a Chairperson of the Board, Vice Chairperson and one or more Vice Presidents, Assistant Treasurers or Assistant Clerks. Any two or more offices may be held by the same person. The Chairperson and Vice Chairperson of the Board shall be elected by and from the Trustees, but other officers of the Corporation need not be Trustees or Members. If required by the Trustees, any officer shall give the Corporation a bond for the faithful performance of his or her duties in such amount and with such surety or sureties as shall be satisfactory to the Trustees.

B. *Tenure.* Except as otherwise provided by law, by the Articles of Organization or by these Bylaws, the President, Treasurer, and all other officers shall hold office until the first meeting of the Board following the annual meeting of Members and thereafter, until his or her successor is chosen and qualified.

C. *Resignation.* Any officer may resign by delivering his or her written resignation to the Corporation at its principal office or to the President or Clerk and such resignation shall be effective upon receipt unless it is specified to be effective at some other time or upon the happening of some other event.

D. *Removal.* The Board may remove any officer with or without cause by a vote of a majority of the entire number of Trustees then in office, at a meeting of the Board called for that purpose and for which notice of the purpose thereof has been given, provided that an officer may be removed for cause only after having an opportunity to be heard by the Board at a meeting of the Board at which a quorum is personally present and voting.

E. *Vacancy.* A vacancy in any office may be filled for the unexpired balance of the term by vote of a majority of the Trustees present at any meeting of Trustees at which a quorum is present or by written consent of all of the Trustees, if less than a quorum of Trustees shall remain in office.

F. *Chairperson.* The Chairperson shall have such powers and duties as may be determined by the Board and, unless otherwise determined by the Board, shall serve in that capacity for a term coterminous with his or her term as Trustee.

G. *Vice Chairperson.* The Vice Chairperson shall perform the duties and exercise the powers of the Chairperson in the absence or disability of the Chairperson, and shall perform such other duties and possess such other powers as may be determined by the Board. Unless otherwise determined by the Board, the Vice Chairperson shall serve for a one-year term.

H. *Director.* The Director shall be the chief operating officer and, unless otherwise voted by the Trustees, the chief executive officer of the Corporation. The Director shall, subject to the direction of the Trustees, have general supervision of the Laboratory and control of the business of the Corporation. At the annual meeting, the Director shall submit a report of the operations of the Corporation for such year and a statement of its affairs, and shall, from time to time, report to the Board all matters within his or her knowledge which the interests of the Corporation may require to be brought to its notice.

1. *Deputy Director.* The Deputy Director, if any, or if there shall be more than one, the Deputy Directors in the order determined by the Trustees, shall, in the absence or disability of the Director, perform the duties and exercise the powers of the Director and shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

J. *President.* The President shall have the powers and duties as may be vested in him or her by the Board.

K. *Treasurer and Assistant Treasurer.* The Treasurer shall, subject to the direction of the Trustees, have general charge of the financial affairs of the Corporation, including its long-range financial planning, and shall cause to be kept accurate books of account. The Treasurer shall prepare a yearly report on the financial status of the Corporation to be delivered at the annual meeting. The Treasurer shall also prepare or oversee all filings required by the Commonwealth of Massachusetts, the Internal Revenue Service, or other Federal and State Agencies. The account of the Treasurer shall be audited annually by a certified public accountant.

The Assistant Treasurer, if any, or if there shall be more than one, the Assistant Treasurers in the order determined by the Trustees, shall, in the absence or disability of the Treasurer, perform the duties and exercise the powers of the Treasurer, shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

L. *Clerk and Assistant Clerk.* The Clerk shall be a resident of the Commonwealth of Massachusetts, unless the Corporation has designated a resident agent in the manner provided by law. The minutes or records of all meetings of the Trustees and Members shall be kept by the Clerk who shall record, upon the record books of the Corporation, minutes of the proceedings at such meetings. He or she shall have custody of the record books of the Corporation and shall have such other powers and shall perform such other duties as the Trustees may, from time to time, prescribe.

The Assistant Clerk, if any, or if there shall be more than one, the Assistant Clerks in the order determined by the Trustees, shall, in the absence or disability of the Clerk, perform the duties and exercise the powers of the Clerk and shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

In the absence of the Clerk and an Assistant Clerk from any meeting, a temporary Clerk shall be appointed at the meeting.

M. *Other Powers and Duties.* Each officer shall have in addition to the duties and powers specifically set forth in these Bylaws, such duties and powers as are customarily incident to his or her office, and such duties and powers as the Trustees may, from time to time, designate.

ARTICLE VII—AMENDMENTS

These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

ARTICLE VIII—INDEMNITY

Except as otherwise provided below, the Corporation shall, to the extent legally permissible, indemnify each person who is, or shall have been, a Trustee, director or officer of the Corporation or who is serving, or shall have served at the request of the Corporation as a Trustee, director or officer of another organization in which the Corporation directly or indirectly has any interest as a shareholder, creditor or otherwise, against all liabilities and expenses (including judgments, fines, penalties, and reasonable attorneys' fees and all amounts paid, other than to the Corporation or such other organization, in compromise or settlement) imposed upon or incurred by any such person in connection with, or arising out of, the defense or disposition of any action, suit or other proceeding, whether civil or criminal, in which he or she may be a defendant or with which he or she may be threatened or otherwise involved, directly or indirectly, by reason of his or her being or having been such a Trustee, director or officer.

The Corporation shall provide no indemnification with respect to any matter as to which any such Trustee, director or officer shall be finally adjudicated in such action, suit or proceeding not to have acted in good faith in the reasonable belief that his or her action was in the best interests of the Corporation. The Corporation shall provide no indemnification with respect to any matter settled or compromised unless such matter shall have been approved as in the best interests of the Corporation, after notice that indemnification is involved, by (i) a disinterested majority of the Board of the Executive Committee, or (ii) a majority of the Members.

Indemnification may include payment by the Corporation of expenses in defending a civil or criminal action or proceeding in advance of the final disposition of such action or proceeding upon receipt of an undertaking by the person indemnified to repay such payment if it is ultimately determined that such person is not entitled to indemnification under the provisions of this Article VIII, or under any applicable law.

As used in the Article VIII, the terms "Trustee," "director," and "officer" include their respective heirs, executors, administrators and legal representatives, and an "interested" Trustee, director or officer is one against whom in such capacity the proceeding in question or another proceeding on the same or similar grounds is then pending.

To assure indemnification under this Article VIII of all persons who are determined by the Corporation or otherwise to be or to have been "fiduciaries" of any employee benefits plan of the Corporation which may exist, from time to time, this Article VIII shall be interpreted as follows: (i) "another organization" shall be deemed to include such an employee benefit plan, including without limitation, any plan of the Corporation which is governed by the Act of Congress entitled "Employee Retirement Income Security Act of 1974," as amended, from time to time, ("ERISA"); (ii) "Trustee" shall be deemed to include any person requested by the Corporation to serve as such for an employee benefit plan where the performance by such person of his or her duties to the Corporation also imposes duties on, or otherwise involves services by, such person to the plan or participants or beneficiaries of the plan; (iii) "fines" shall be deemed to include any excise tax plan pursuant to ERISA; and (iv) actions taken or omitted by a person with respect to an employee benefit plan in the performance of such person's duties for a purpose reasonably believed by such person to be in the interest of the participants and beneficiaries of the plan shall be deemed to be for a purpose which is in the best interests of the Corporation.

The right of indemnification provided in this Article VIII shall not be exclusive of or affect any other rights to which any Trustee, director or officer may be entitled under any agreement, statute, vote of Members or otherwise. The Corporation's obligation to provide indemnification under this Article VIII shall be offset to the extent of any other source of indemnification of any otherwise applicable insurance coverage under a policy maintained by the Corporation or any other person. Nothing contained in the Article shall affect any rights to which employees and corporate personnel other than Trustees, directors or officers may be entitled by contract, by vote of the Board or of the Executive Committee or otherwise.

ARTICLE IX—DISSOLUTION

The consent of every Trustee shall be necessary to effect a dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Trustees then in office in accordance with the laws of the Commonwealth of Massachusetts.

ARTICLE X—MISCELLANEOUS PROVISIONS

A. *Fiscal Year.* Except as otherwise determined by the Trustees, the fiscal year of the Corporation shall end on December 31st of each year.

B. *Seal.* Unless otherwise determined by the Trustees, the Corporation may have a seal in such form as the Trustees may determine, from time to time.

C. *Execution of Instruments.* All checks, deeds, leases, transfers, contracts, bonds, notes and other obligations authorized to be executed by an officer of the Corporation in its behalf shall be signed by the Director or the Treasurer except as the Trustees may generally or in particular cases otherwise determine. A certificate by the Clerk or an Assistant Clerk, or a temporary Clerk, as to any action taken by the Members, Board of Trustees or any officer or representative of the Corporation shall as to all persons who rely thereon in good faith be conclusive evidence of such action.

D. *Corporate Records.* The original, or attested copies, of the Articles of Organization, Bylaws and records of all meetings of the Members shall be kept in

Massachusetts at the principal office of the Corporation, or at an office of the Corporation's Clerk or resident agent. Said copies and records need not all be kept in the same office. They shall be available at all reasonable times for inspection by any Member for any proper purpose, but not to secure a list of Members for a purpose other than in the interest of the applicant, as a Member, relative to the affairs of the Corporation.

E. *Articles of Organization.* All references in these Bylaws to the Articles of Organization shall be deemed to refer to the Articles of Organization of the Corporation, as amended and in effect, from time to time.

F. *Transactions with Interested Parties.* In the absence of fraud, no contract or other transaction between this Corporation and any other corporation or any firm, association, partnership or person shall be affected or invalidated by the fact that any Trustee or officer of this Corporation is pecuniarily or otherwise interested in or is a director, member or officer of such other corporation or of such firm, association or partnership or in a party to or is pecuniarily or otherwise

interested in such contract or other transaction or is in any way connected with any person or person, firm, association, partnership, or corporation pecuniarily or otherwise interested therein; provided that the fact that he or she individually or as a director, member or officer of such corporation, firm, association or partnership in such a party or is so interested shall be disclosed to or shall have been known by the Board of Trustees or a majority of such Members thereof as shall be present at a meeting of the Board of Trustees at which action upon any such contract or transaction shall be taken; any Trustee may be counted in determining the existence of a quorum and may vote at any meeting of the Board of Trustees for the purpose of authorizing any such contract or transaction with like force and effect as if he/she were not so interested, or were not a director, member or officer of such other corporation, firm, association or partnership, provided that any vote with respect to such contract or transaction must be adopted by a majority of the Trustees then in office who have no interest in such contract or transaction.





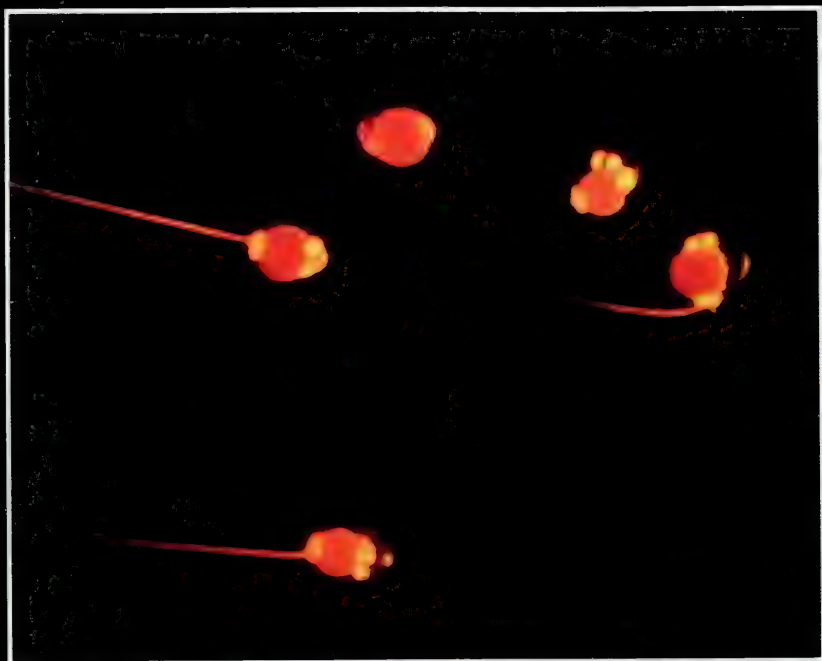
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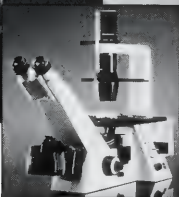
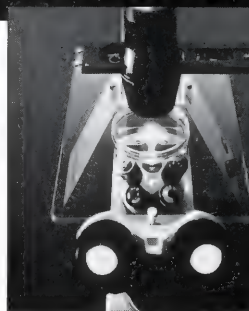
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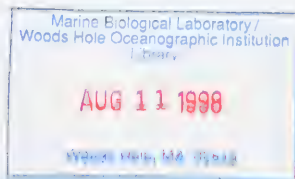
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Cover

Gossypol-treated sperm from *Spisula solidissima*, the surf clam, fluoresce when observed with real-time confocal microscopy. Sperm were stained with Eosin-B. See the Short Reports by Inoué *et al.*, Tran *et al.*, and Burgos *et al.*, this issue.

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INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

Very short, especially topical papers (less than 9 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled "Research Notes." A Research Note in *The Biological Bulletin* follows the format of similar notes in *Nature*. It should open with a summary paragraph of 150 to 200 words comprising the introduction and the conclusions. The rest of the text should continue on without subheadings, and there should be no more than 30 references. References should be referred to in the text by number, and listed in the Literature Cited section in the order that they appear in the text. Unlike references in *Nature*, references in the Research Notes section should conform in punctuation and arrangement to the style of recent issues of *The Biological Bulletin*. Materials and Methods should be incorporated into appropriate figure legends. See the article by Lohmann *et al.* (October 1990, Vol. 179: 214–218) for sample style. A Research Note will usually appear within two months after its acceptance.

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Chemosensory Activation of an Antennular Grooming Behavior in the Spiny Lobster, *Panulirus argus*, Is Tuned Narrowly to L-Glutamate

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Abstract. Antennular grooming behavior (AGB) is a stereotyped behavior in crustaceans in which the first pair of antennae, the major olfactory organs, are clasped and wiped repetitively by the third maxillipeds, which also serve as feeding appendages. AGB apparently functions to clear away accumulating debris on or between the antennular aesthetascs (olfactory sensilla). The purpose of this research was to determine whether AGB can be activated by chemicals commonly found in food odors. Lobsters were presented, via headset or handheld pipette, with 27 chemicals found in their food. One chemical, L-glutamate, evoked very high frequencies of wiping. Most chemicals tested were not stimulatory and only a few were weakly stimulatory (adenosine-5'-monophosphate, glycine, D-glutamate). This is surprising because previous studies have shown that other behaviors (antennular flick, search) can be evoked by a much broader array of chemicals found in food odorants. On the basis of these results, we propose that chemosensory neurons that specifically detect L-Glu activate AGB through a recently described non-olfactory pathway. Furthermore, we propose that the role of L-Glu in evoking AGB is based on its electrostatic properties. Because it has a high probability of electro-

static adherence to the antennular cuticle, L-Glu is a sensitive indicator of fouling by food-associated chemicals and thus an appropriate compound to stimulate antennular grooming.

Introduction

Odorant access and removal from receptors are equally important for effective chemical detection. To facilitate these two processes, animals have evolved short- and long-term mechanisms that include elimination of stagnant boundary layers, alteration and internalization of chemostimulants, and grooming of chemosensory organs. These mechanisms maintain the sensitivity of the receptor cell and, in the case of grooming, the structural integrity of the chemosensory organ.

Crustaceans are good models in which to investigate chemoreception because they respond to chemostimulants with highly stereotypical and quantifiable behaviors. Chemical cues regulate many components of crustacean behavior including food recognition (Hazlett, 1968; Carr, 1978; Carr, 1982; Schembri, 1981; Fine-Levy *et al.*, 1988), courtship behavior (Atema and Engstrom, 1971; Dunham, 1978; Atema *et al.*, 1979; Gleeson, 1980), and predator avoidance (Mackie and Grant, 1974).

Electrophysiological recordings of receptor neurons (*e.g.*, Derby and Ache, 1984; Anderson and Ache, 1985) and behavioral studies employing ablation techniques (Derby and Atema, 1982; Fine-Levy *et al.*, 1988; Daniel and Derby, 1991) have shown that antennules are the primary organs for detecting odors. Therefore, mechanisms that enhance odorant access to and removal from the perireceptor environment of the antennules are essential to crustacean behavior. Such mechanisms include antennular flicking (Snow, 1973; Gleeson *et al.*, 1993),

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Abbreviations: antennular grooming behavior (AGB), olfactory receptor neurons (ORNs), adenosine-5'-monophosphate (AMP), D- & L-alanine (D- & L-Ala), L-arginine (L-Arg), L-asparagine (L-Asn), L-aspartate (L-Asp), betaine (Bet), L-cysteine (L-Cys), D- & L-glutamate (D- & L-Glu), glycine (Gly), L-histidine (L-His), L-hydroxyproline (L-Hyp), L-isoleucine (L-Ile), L-lactate (L-Lac), L-leucine (L-Leu), L-lysine (L-Lys), L-methionine (L-Met), ammonium chloride (NH₄Cl), N-methyl-D-aspartate (NMDA), L-phenylalanine (L-Phe), L-proline (L-Pro), L-serine (L-Ser), L-succinate (L-Suc), taurine (Tau), L-threonine (L-Thr), L-valine (L-Val), artificial seawater (ASW).

biochemical removal of stimuli (Gleeson *et al.*, 1987; Carr *et al.*, 1989), and antennular grooming (Bauer, 1989).

Antennular flicking, which occurs at frequencies of 0.5 to 2 Hz in *Panulirus argus* (Schmitt and Ache, 1979), is defined as the vertical deflection of the lateral filament to a position nearly contacting the medial filament (Zimmer-Faust *et al.*, 1984). Antennular flicking reduces the stagnant boundary layers of seawater created by the dense tufts of aesthetascs (Snow, 1973; Gleeson *et al.*, 1993) which are the sensilla housing the dendritic processes of hundreds of olfactory receptor neurons (ORNs) (Grünert and Ache, 1988). This removal mechanism uses movements of the antennules to splay apart the aesthetasc hairs, allowing water to rush in and displace the water collected during the previous flick. Flicking is analogous to a vertebrate sniff (Schmitt and Ache, 1979) because sniffing flushes air from the upper nasal cavity, the location of the olfactory epithelium. In *P. argus*, flicking is activated in a dose-dependent manner by a broad range of chemical stimuli detected by the antennules (Daniel and Derby, 1991).

The aesthetascs of *P. argus* contain membrane-bound enzymes and transporters that serve to eliminate specific chemical stimuli including nucleotides and amino acids (Trapido-Rosenthal *et al.*, 1988; Carr *et al.*, 1989). Ecto-nucleotidases catalyze the dephosphorylation of adenine nucleotides, creating an odorant different from the one that initially entered the receptor environment. The end product of dephosphorylation, adenosine, a molecule for which there is little receptor sensitivity, is internalized by a specific uptake system (Trapido-Rosenthal *et al.*, 1987). In addition to the adenosine uptake system, an uptake system for taurine has been identified (Gleeson *et al.*, 1987) and uptake systems for other amino acids, including L-glutamate, also exist (Trapido-Rosenthal *et al.*, 1988; Carr *et al.*, 1989). Prolonged stimulation of ORNs limits the temporal and spatial resolution of "patchy" olfactory environments (Atema *et al.*, 1989). Quick elimination of odorants by these biochemical removal mechanisms as well as by antennular flicking enhances receptor sensitivity.

The least studied mechanism for facilitating odorant removal and maintaining receptor accessibility is antennular grooming behavior (AGB). Although AGB is common in many crustaceans, very little is known about which stimuli activate it (Bauer, 1989). This behavior consists of an antennular deflection downward, permitting the lateral and medial antennular filaments to be grasped by the third maxillipeds (paired appendages on either side of the mouth) and pulled repeatedly through the setal combs of the maxillipeds. The resulting action facilitates removal of material that has accumulated on or between the aesthetascs (Snow, 1973; Bauer, 1989). Fouling is a recurring problem for crustaceans because both the exoskeleton and specialized structures such as gills and anten-

nules are favorable substrates for microbes and detritus. If this material is not removed, chitinous microorganisms can damage the exoskeleton and the presence of other fouling organisms can cause respiratory and sensory impairment. When a shrimp, *Heptacarpus pictus*, was experimentally prevented from grooming the antennules, extensive structural damage of the aesthetascs occurred (Bauer, 1977). Therefore, anything that enhances the level of microbial fouling is detrimental to the structural integrity of the antennule and, presumably, to its functional role as a chemoreceptor organ.

Although fouling of the antennules occurs continually, feeding may result in particularly high rates of accumulation of debris. By providing a nutrient-rich substrate, this debris facilitates microbial colonization. Results of studies of several crustacean species show that AGB can be elicited in response to compounds typically found in food (Snow, 1973; Zimmer-Faust *et al.*, 1984). In this paper, we examine which chemicals found in the food of *P. argus* stimulate the release of AGB. Compounds found in food, including amino acids, nucleotides, organic acids and ammonium (Carr and Derby, 1986) were tested. Results of behavioral assays revealed that only one amino acid, L-glutamate (L-Glu), evoked AGB with great frequency. Even analogues of L-Glu (D-glutamate, L-aspartate, N-methyl-D-aspartate) were not excitatory. On the basis of these results, we propose that chemosensory neurons that specifically detect L-Glu activate AGB through a recently described non-olfactory pathway (Schmidt *et al.*, 1992; Schmidt and Ache, 1996). Furthermore, we propose that L-Glu evokes AGB because the high probability of electrostatic adherence to the antennular cuticle makes it a sensitive indicator of fouling by food-associated chemicals.

Materials and Methods

Source and maintenance of lobsters

Spiny lobsters (55 to 70 mm carapace length) were obtained from the Florida Keys Regional Marine Laboratory in Long Key, Florida, and maintained in separate 80-l aquaria (one lobster/aquarium). Aquaria were lined with crushed coral; filled with aerated, recirculating Instant Ocean (specific gravity, 1.021–1.023); and equipped with gravel-bottom filter systems. Lobsters were fed scallop or shrimp daily *ad libitum* and the uneaten food was removed after 1 h. The light:dark cycle was 12:12 and the ambient temperature was maintained between 25°–27°C. Red light (25 W, ceramic-coated light bulbs) was provided during the dark cycle.

Chemical Stimuli

The following compounds were used as stimuli: adenosine-5'-monophosphate (AMP), D- & L-alanine (D- & L-

Ala), L-arginine (L-Arg), L-asparagine (L-Asn), L-aspartate (L-Asp), betaine (Bet), D- & L-glutamate (D- & L-Glu), glycine (Gly), L-histidine (L-His), L-hydroxyproline (L-Hyp), L-isoleucine (L-Ile), L-lactate (L-Lac), L-leucine (L-Leu), L-lysine (L-Lys), L-methionine (L-Met), ammonium chloride (NH_4Cl), N-methyl-D-aspartate (NMDA), L-phenylalanine (L-Phe), L-proline (L-Pro), L-serine (L-Ser), L-succinate (L-Suc), taurine (Tau), L-threonine (L-Thr) and L-valine (L-Val). Stock solutions (10 mM, pH 8.1) of all the stimuli were prepared in artificial seawater (ASW) (Cavanaugh, 1964). L-cysteine (L-Cys) was prepared on the day of testing to prevent precipitation from freezing; all others were stored at -70°C until used. With the exception of D-Ala, D-Glu (a stereoisomer of Glu), NH_4Cl , and NMDA (an agonist of a subclass of glutamate receptors (Schoepp, 1994)), these compounds were identified by Carr and Derby (1986) as components of prey extracts. On the day of testing, appropriate stock solutions were thawed and diluted to either 5 mM or 0.5 mM with ASW.

Experimental design

The 26 compounds listed above were each assayed at 0.5 mM. To ensure that the lobsters would not become desensitized, we presented these chemicals in five separate trials (maximum of 10 chemicals per trial including ASW & L-Glu) so that no experiment lasted longer than 4 h. We subsequently tested 10 of these compounds (in three separate trials) at 5.0 mM. In previous trials, these 10 chemicals had elicited either a highly significant response (*i.e.*, one that was significantly greater than the response toward ASW) or the response was greater than the response toward ASW for a majority of lobsters. The exception was NMDA, which was assayed at both concentrations even though it failed to meet the criteria. Finally, a series of concentrations of L-Glu (0.01, 0.05, 0.08, and 0.1 mM) and three other compounds (AMP, Gly, D-Glu at 0.5, 1.0, 5.0 and 10.0 mM) shown to elicit significant responses at 5.0 mM were tested. Each of the four compounds was assayed in a separate trial. All trials included ASW as a control stimulus and 0.5 mM L-Glu, which preliminary experiments showed to be very effective in evoking AGB, as a response standard.

Presentation of stimuli

Experimental trials were blind in that the experimenter did not know the order in which the chemicals were presented. Each stimulus was administered to six lobsters, except where noted in Results, in triplicate tests. Two methods of stimulus presentation were employed: automated and handheld pipette. For the first two trials using stimuli at 0.5 mM, presentation of stimuli was automated via a headset apparatus as described previously in Daniel and Derby (1988). A 13-mm diameter acrylic rod was

attached by hook and loop fasteners to the rostrum. A bent glass rod (5-mm diameter) was glued to the rostrum attachment with cyanoacrylate quick-bonding nontoxic glue. This provided support for the tubing through which the stimulus was introduced (1-mm inner diameter, attached to the rod with cable ties) and allowed test solutions to be injected at a constant distance in the vicinity of the antennules. A peristaltic pump was used to deliver all solutions. To desensitize the animals to the mechanical stimulus, tank water was pumped continuously, via plastic flexible tubing, from the tank through the stimulus-introduction tubing. Each test solution (5-ml in a 10-ml plastic syringe) was injected into the tubing through a two-way stopcock valve. The flow rate of the pump was maintained at $10\text{-ml}\cdot\text{min}^{-1}$. In subsequent trials, stimuli were presented via a handheld 5-ml pipette. The tip of the pipette was gently placed in the vicinity of the antennules. This method appeared to elicit a consistently greater magnitude of AGB from the lobster (Fig. 1). All trials were videotaped, beginning 0.25 min before each stimulus was presented and continuing for up to 2 min afterward.

Data analysis

The magnitudes of the AGB responses in all experiments were determined from videotapes. Pre-stimulus wipe rates were determined by counting the number of wipes that occurred in the 0.25-min period before stimulus presentation. Post-stimulus wipe rates were determined by recording the number of wipes that occurred for 1 min after stimulus presentation. Wipe rates were therefore defined as the post-stimulus response rate minus the pre-stimulus response rate. The three wipe rates ($\text{wipes}\cdot\text{min}^{-1}$) counted for each stimulus per trial were averaged and reported as the mean wipe rate.

In most cases, data did not meet the assumptions necessary for parametric statistical tests; therefore, nonparametric tests were used. Friedman's repeated measures tests on ranks was used to compare responses to chemical stimuli (Sigmastat, Jandel Scientific). Where statistically significant differences were found, pairwise comparisons were performed using the Student-Newman-Keuls (S-N-K) test adapted for ranked data. For the concentration series experiments, least-squares regression analyses were performed on log-transformed concentrations of each stimulus vs. wipe rates standardized to the L-Glu (0.5 mM) response.

Results

Responses to compounds at 0.5 mM

Of the compounds presented at 0.5 mM, L-Glu was by far the most effective compound at eliciting AGB. In trials #1–#4, reported in Figure 1, L-Glu produced significantly

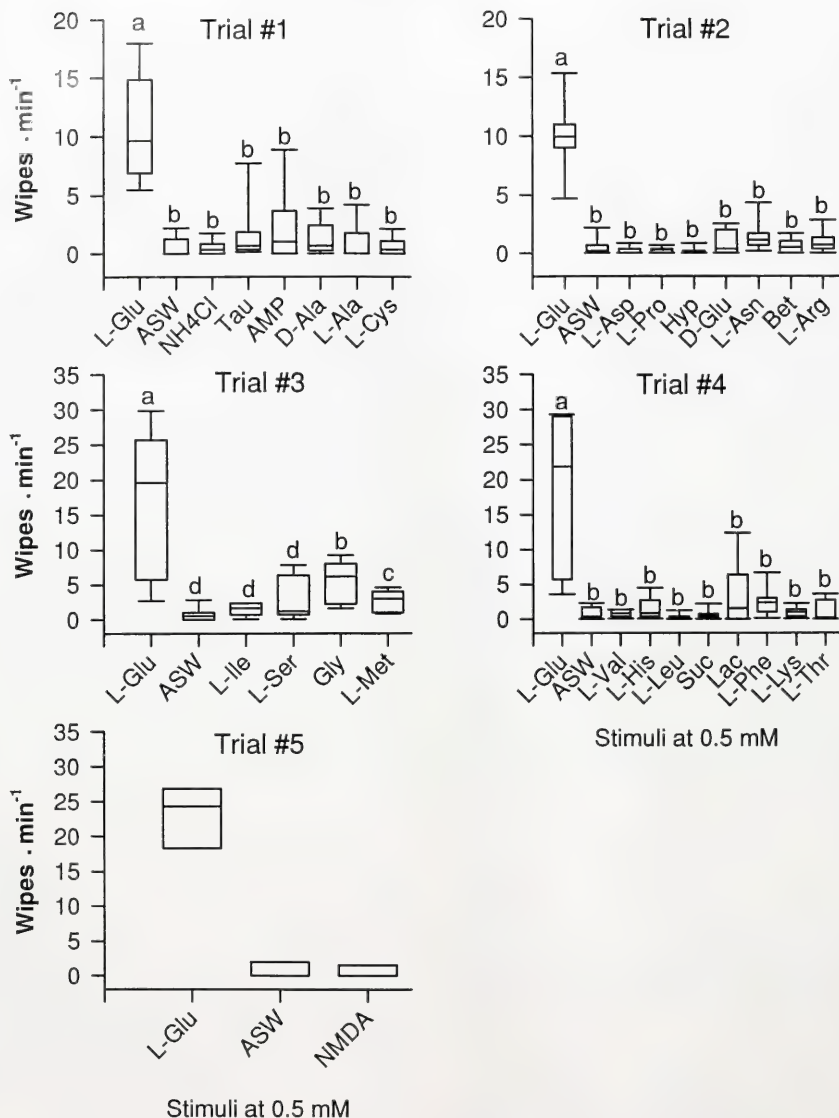


Figure 1. Magnitude of AGB (number of wipes $\cdot$ min⁻¹) towards single stimuli at 0.5 mM. Nine lobsters were tested in trials #1 and #2, six in trials #3 and #4, and three in trial #5. Each box plot shows the distribution of lobster responses. The 25th and 75th percentiles are shown as the lower and upper limits of the box and the 50th percentile as the line within the box. The caps on vertical lines show the 10th and 90th percentiles. Stimuli with significantly different wipe rates within a trial are indicated by different letters above box plots. See text for details of statistical results.

higher wipe rates than all other stimuli tested at 0.5 mM and ASW (Fig 1; Friedman repeated measures ANOVA; trial #1, $\chi^2 = 29.7$, $n = 9$, $P = 0.0001$; trial #2, $\chi^2 = 34.6$, $n = 9$, $P = 0.0001$; trial #3, $\chi^2 = 24.4$, $n = 6$, $P = 0.0002$; trial #4, $\chi^2 = 23.1$, $n = 6$, $P = 0.006$; S-N-K pairwise comparisons test for ranked data, $P < 0.05$). Two compounds, Gly and L-Met (trial #3), elicited responses that were significantly greater than ASW (S-N-K pairwise comparisons test for ranked data, $P < 0.05$). Neither the stereoisomer of L-Glu, D-Glu, nor the analogue, L-Asp (sidechain has one less carbon), elicited responses greater than those toward ASW in trial #3 (S-N-K pairwise comparisons test for ranked data, $P > 0.05$). In addition, NMDA did not elicit significant responses (trial #5, $\chi^2 = 4.91$, $n = 3$, $P = 0.194$). However, none of the compounds tested in this trial, including L-Glu, were significantly different from ASW. This is probably because the small sample size used in this experiment resulted in a very low-power test. However, all three lobsters tested responded much more to L-Glu than to ASW or NMDA at 0.5 mM.

Responses to compounds at 5 mM

Responses to L-Glu at 0.5 mM were significantly higher than responses to test stimuli when presented at 5.0 mM (Fig. 2; Friedman's repeated measures ANOVA; trial #1, $\chi^2 = 43.1$, $n = 9$, $P = 0.0001$; trial #2, $\chi^2 = 24.7$, $n = 6$, $P = 0.0002$; trial #3, $\chi^2 = 10.4$, $n = 6$, $P = 0.0055$; S-N-K pairwise comparisons test for ranked data, $P < 0.05$). Six compounds, L-Asn, L-Cys, AMP, and Tau in trial #1, Gly and D-Glu in trial #2, evoked wipe rates significantly greater than those for ASW (S-N-K pairwise comparisons test for ranked data, $P < 0.05$). NMDA produced no significant response at 5.0 mM (trial #3, S-N-K pairwise comparisons test for ranked data, $P > 0.05$).

Responses to concentration series

The magnitude of AGB towards L-Glu increased as a linear function of the log of its concentration (Fig. 3, least-squares regression, $F = 86.6$, $n = 30$, $P < 0.001$, $r^2 = 0.76$, standardized wipes $\cdot \text{min}^{-1} = 107.7 + (53.1 \cdot \log[\text{concentration}])$ and were at least 100 times more effective than any of the other chemicals across the range of concentrations tested. Only the AMP concentration series yielded a significant regression (least-squares regression, $F = 22.6$, $n = 26$, $P < 0.0001$, $r^2 = 0.55$, standardized wipes $\cdot \text{min}^{-1} = 7.26 + (39.5 \cdot \log[\text{concentration}])$. From the linear regression equations, the concentrations needed to achieve 25% and 50% maximal responses were 0.03 and 0.08 mM, respectively, for L-Glu, and 3.0 and 14 mM, respectively, for AMP. Although the linear regression equations for Gly and D-Glu were not

significant, the 50% maximal responses based on visual inspection were at least 10 mM.

Discussion

AGB specificity to L-glutamate: implications for sensory-motor integration

Our results show that, unlike other behaviors studied in *P. argus* (Fine-Levy *et al.*, 1988, 1989; Daniel and Derby, 1988; Fine-Levy and Derby, 1991, 1992; Lynn *et al.*, 1994), AGB is elicited almost exclusively by one chemical, the L-enantiomer of glutamate (L-Glu). L-Glu was at least 100 times more effective in eliciting AGB than were AMP, Gly, and D-Glu, the next-best single stimuli. Furthermore, the D-enantiomer of L-Glu (D-Glu), a structural analogue of L-Glu (L-Asp), and an agonist of a major class of glutamate receptors (NMDA), either failed to activate AGB (L-Asp, NMDA) or were only weakly effective at high concentrations (D-Glu). We propose that elicitation of AGB requires sensory input from a specific class of chemosensory neurons narrowly tuned to L-Glu.

According to electrophysiological studies, ORNs in *P. argus* are narrowly tuned to specific chemical stimuli and can be classified by best-compound. "Best" cells have been identified for AMP, ATP, Cys, Bet, Glu, NH_4Cl , and Tau (Derby and Ache, 1984; Carr *et al.*, 1986; Derby *et al.*, 1991; Daniel *et al.*, 1994). Similar tuning characteristics have been identified for olfactory and non-olfactory chemosensitive neurons distributed on second antennae, antennules, maxillipeds, and legs of *Homarus americanus* (Johnson *et al.*, 1985; Tierney *et al.*, 1988; Corotto *et al.*, 1992; Voigt *et al.*, 1997). Biochemical receptor-binding assays of antennules of *P. argus* have identified independent olfactory receptor sites for AMP, Tau (Olson *et al.*, 1992; Olson and Derby, 1995; Sung *et al.*, 1996), both stereoisomers of Ala (Michel *et al.*, 1993), and L-Glu (Burgess *et al.*, 1994).

Compounds that weakly evoke AGB may do so by activating L-Glu-best neurons. Electrophysiological studies of ORNs showed that responses to next-best stimuli were generally 100-fold less than to the best compound (Daniel *et al.*, 1994). Hence the amino acids Gly and D-Glu, and the nucleotide, AMP, are possibly "next-best" stimulants that weakly activate AGB via L-Glu-best cells. However, the response spectra of AGB and of ORNs sensitive to L-Glu are not entirely consistent. NMDA and L-Cys serve as partial agonists and antagonists for chemoreceptors presumed to be ORNs sensitive to glutamate (Burgess and Derby, 1995). Since AGB was elicited by L-Glu but not NMDA or L-Cys even at 5 mM, it is possible that non-olfactory neurons mediate AGB.

How might such a specific stimulus lead to activation of AGB? There appear to be two antennular chemosensory

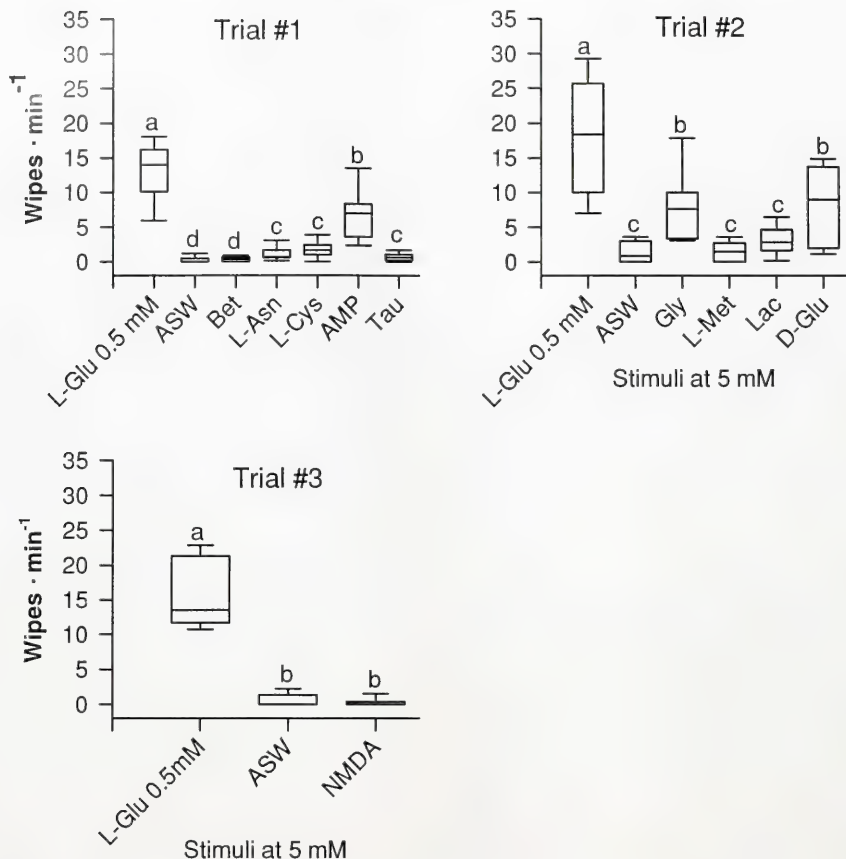


Figure 2. Magnitude of AGB (number of wipes · min⁻¹) towards L-Glu at 0.5 mM and other single stimuli at 5 mM. Nine lobsters were tested in trial #1 and six in trials #2 and #3. Each box plot shows the distribution of lobster responses. The 25th and 75th percentiles are shown as the lower and upper limits of the box and the 50th percentile as the line within the box. The caps on vertical lines show the 10th and 90th percentiles. Stimuli with significantly different wipe rates within a trial are indicated by different letters above box plots. See text for details of statistical results.

processing pathways in the brain of *P. argus*, an olfactory and a non-olfactory pathway. In the olfactory pathway, ORNs with dendrites located within the aesthetascs on the distal half of the lateral filament of the antennules project to the olfactory lobe (Schmidt and Ache, 1992). In the non-olfactory pathway, chemosensory neurons (as well as mechanosensory neurons) with dendrites in non-aesthetasc sensilla on lateral and medial filaments of the antennules terminate exclusively in the lateral antennular neuropil (LAN) and the medial antennular neuropil

(MAN) in the brain (Schmidt *et al.*, 1992; Schmidt and Ache, 1996). Antennular motoneurons also branch in these neuropils. Similarly, in the blue crab, *Callinectes sapidus*, motoneurons that control such activities as antennular withdrawal and flick originate in these regions (Roye, 1989, 1994).

We propose that the non-olfactory pathway may represent a fairly direct reflexive route for eliciting AGB. According to this hypothesis, non-olfactory chemosensory neurons tuned to L-Glu synapse in the LAN, in the MAN,

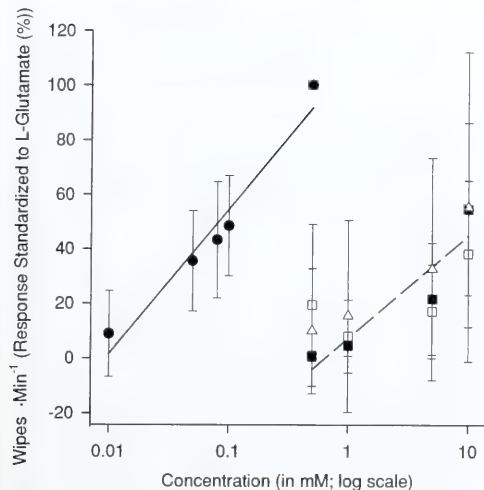


Figure 3. Dose response curves for magnitude of AGB to L-Glu, AMP, Gly, and D-Glu. AGB is expressed as wipes $\cdot$ min $^{-1}$ standardized to the responses to L-Glu at 0.5 mM. Dose-response curves fit by linear regression and found to be statistically significant are shown for AMP (dashed) and L-Glu (solid). Symbols (Δ = D-Glu, $\blacksquare$ = AMP, $\bullet$ = L-Glu, $\square$ = Gly) show the means at each log-transformed concentration, and capped bars show ± 1 standard deviation unit. See text for details of statistical results.

or in both. We suggest that the motor program for AGB resides in these neuropils. Although the tuning of these chemosensory neurons has not been studied in *P. argus*, in *H. americanus* non-olfactory chemosensory neurons, like ORNs have been shown to be tuned narrowly (Voigt and Atema, 1992). This hypothesis may explain the differences in responses of AGB and of presumed ORNs towards NMDA and L-Cys (Burgess and Derby, 1995) discussed above.

Ablation studies of both lateral and medial filaments performed in our laboratory showed that the antennules are the primary sources of the chemosensory input that evokes AGB (Barbato *et al.*, 1996). Ablation of specific regions of the antennules will provide important evidence as to whether AGB is driven by olfactory or non-olfactory input. AGB in *P. argus* provides a useful model for understanding how specific chemosensory information can drive a fairly complex, stereotyped motor program.

Functional significance of L-glu evoked AGB

If chemosensory-mediated AGB in the spiny lobster is a mechanism for removing antennular debris—as previous studies on other crustaceans suggest (*e.g.*, Bauer,

1977)—it may be that L-Glu is a sensitive indicator of potential biofouling. L-Glu is a common constituent of extracts of typical food (Carr and Derby, 1986; Carr *et al.*, 1996). For example, 5 mM complex mixtures mimicking shrimp, mullet, crab, and oyster extracts contain L-Glu concentrations of 0.025, 0.026, 0.034, and 0.06 mM, respectively (Carr and Derby, 1986). According to the dose-response curve shown in Figure 3, the predicted AGB response at these concentrations is 23%, 23%, 30%, and 43%, respectively, of the maximum standardized L-Glu response. In fact, we have observed significant levels of AGB elicited by 5 mM shrimp and oyster mixtures (Barbato *et al.*, 1996). If lobsters encounter food effluents at these concentrations, which odor plume studies (Moore and Atema, 1991) indicate would probably occur within the immediate vicinity of a food source, there is a good chance that they may be induced by L-Glu to groom their antennules.

In addition, L-Glu is very “sticky” compared to other compounds found in complex mixtures mimicking natural prey extracts. At the pH of seawater, 8.1, L-Glu has three charged functional groups: two negative carboxyl groups, and one positive amino group (Lehninger *et al.*, 1993). Conversely, most of the remaining chemicals found in complex mixtures have a maximum of two charged functional groups. For example, of the 33 chemicals in complex mixtures mimicking oyster extract and the 29 in complex mixtures mimicking shrimp extract (Carr and Derby, 1986), only three amino acids (Asp, Lys, Arg) possess a charge orientation similar to that of L-Glu. This charge orientation may make it possible for an electrostatic attraction to be established between these four amino acids and the cuticle of the aesthetasc. The outer portion of crustacean cuticle is composed of spherulitic calcite islands surrounded by a lipoprotein matrix (Roer and Dillaman, 1984), so the amino acid residues found in the matrix may be the site of the attraction. The four amino acids with the special charge orientation would be more likely to stick to the cuticle surrounding the aesthetasc than other compounds.

Of these compounds, only L-Glu is known to elicit significant excitatory activity from ORNs (Derby and Ache, 1984). Similarly, in our study only L-Glu elicited AGB activity. Therefore, because L-Glu can easily adhere to the cuticle, is commonly found in food, and can be detected by chemoreceptors, it is the most sensitive indicator of potential fouling by food. By keying in on this chemical, the lobster may ensure the complete removal of other fouling material, thus preventing the buildup of debris on the antennules.

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Catecholamine-Containing Cells in Larval and Postlarval Bivalve Molluscs

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Abstract. Previous studies have suggested an involvement of catecholamines in the control of several larval behaviors, such as feeding, locomotion, and induction of settling and metamorphosis. In the present study we employed aldehyde-induced, blue-green fluorescence to indicate catecholamines in cells within representatives of two bivalve families, the Pectinidae (*Placopecten magellanicus*) and the Mytilidae (*Mytilus edulis*). Larvae were examined at different stages of development before and also shortly after settlement. The general distribution of fluorescent cells was similar in the two species. By mid-veliger stage, several fluorescent cells and fibers were located along the outer rim of each velar lobe, and a pair of flask-shaped cells was located lateral to the mouth. A single fiber from near the mouth projected to a region beneath the apical tuft. In the pediveliger, the cells by the mouth were joined by an additional two to four fluorescent cells. The developing foot also contained numerous such cells, some of which had processes that penetrated the epithelium on the "sole" and bore ciliated terminals. Fluorescent somata were also located around the edge of the mantle. Centrally projecting fibers appeared to terminate in the pedal and abdominal ganglia, which also contained a few fluorescent somata. After settlement, the velar lobes and resident fluorescent somata disappeared, but fluorescent cells in the foot persisted as this latter organ grew. Fluorescent cells within the developing gill were connected with the abdominal ganglia by means of fibers. Control preparations labeled with antibodies raised against serotonin indicated that the aldehyde-induced fluorescence was not due to the presence of indoleamines. The present study not only confirms previous chromato-

graphic evidence suggesting the presence of catecholamines in the larvae of bivalve molluscs, but also identifies putative neuronal circuits that may control various larval behaviors.

Introduction

Catecholamines have been implicated in many of the physiological processes that occur during the larval development of molluscs. For instance, several lines of evidence suggest that these substances can act as morphogens, directly influencing early development (Voronezhskaya *et al.*, 1992, 1993; Buznikov *et al.*, 1996). Other work suggests roles for catecholamines in the control of ciliary activity (Beiras and Widdows, 1995a) and in the triggering of settling and metamorphosis (Hadfield, 1984; Coon *et al.*, 1985; Voronezhskaya *et al.*, 1992, 1993; Beiras and Widdows, 1995b; but see Pires and Hadfield, 1991). Although chromatographic techniques have indicated the presence of catecholamines in molluscan larvae (Coon and Bonar, 1986), the catecholaminergic neurons and pathways, which have been hypothesized to mediate such processes (Bonar *et al.*, 1990; Beiras and Widdows, 1995a, b), have not previously been identified. In the present report we provide histochemical evidence that many catecholaminergic neurons are located in the velum, foot, mantle, central nervous system, and mouth area of mussel and scallop larvae, and we describe how the distributions of these neurons change with metamorphosis. This work thus provides new foci for the critical testing of hypotheses about the roles of catecholamines in the development of molluscan larvae and provides a basis for comparisons with parallel functions across related phyla (Marsden and Hassessian, 1986; Edwards *et al.*, 1987; Hay-Schmidt, 1990a, b, 1992).

Materials and Methods

Animals

Adult blue mussels (*Mytilus edulis*) were collected from the Aqua Prime aquacultural farm in Ship Harbour, Nova Scotia, and transferred to the Aquatron Seawater Laboratory of Dalhousie University. Spawning was induced by thermal stimulation (8° – 10° C above ambient), and the larvae were incubated in 50-l polyethylene containers for 72 h at 12° C until the straight-hinged (D-stage) veliger was reached. (See Bayne, 1971, for a description of larval development in *M. edulis*.) Larvae were then transferred to 1000-l insulated tanks filled with 12° C, filtered ($0.2\ \mu\text{m}$) seawater, which was changed every 2 or 3 days. The algal food source was *Isochrysis galbana* (clone ISO; Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME), fed at a concentration of 25,000 cells/ml after each water change. Larvae of the sea scallop (*Placopecten magellanicus*) were obtained directly from the Fisheries Resource Development Corporation hatchery in Sandy Cove, Nova Scotia, and were prepared for histochemical study immediately after their delivery to the laboratory. (See Culliney, 1974, and Cragg and Crisp, 1991, for descriptions and staging of larval development in *P. magellanicus*.)

Before relaxation or fixation, the larvae were placed under a dissecting microscope to observe their behavior and general morphology.

Histochemistry

Larvae were collected on a $60\text{-}\mu\text{m}$ -mesh Nitex screen and rinsed into 20-ml vials with filtered ($0.2\ \mu\text{m}$) seawater. Whole larvae were either first relaxed by gradually adding 7.5% (W:V) MgCl_2 to their natural seawater bath until the valves gaped open and the vela were extended (Omori and Ikeda, 1984) or were processed directly using the FaGlu procedure modified from Furness *et al.* (1977). In brief, living larvae were immersed in a solution of 4% paraformaldehyde and 0.55% glutaraldehyde in phosphate-buffered saline (PBS: 50 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 140 mM NaCl, pH 7.4). After 2–18 h, the fixed larvae were placed on glass slides, excess solution was removed with paper wicks, and the slides were desiccated in the dark at room temperature for 4–18 h. A drop of either paraffin oil or methyl salicylate was added before a coverslip was placed over the slide. The mounted slides were viewed through a Leitz Aristoplan microscope equipped for ultraviolet (UV) fluorescence (D filter block providing 355–425 nm excitation and 460 nm long-pass barrier filter) and photographed using either Kodak T-MAX 100 or Ektachrome 400 film. Negative controls were also prepared with no glutaraldehyde added to the fixative. Such preparations exhibited none of the blue-green fluorescence reported in this study.

Similar histochemical techniques are known to specifically induce blue-green fluorescence that is characteristic of catecholamines in a wide variety of animals (Schöler and Armstrong, 1982; Hauser and Koopowitz, 1987; Molist *et al.*, 1993), including molluscs (Croll and Chiasson, 1990). Little or no yellow fluorescence of indoleamines (Furness *et al.*, 1977) was noted in any of the aforementioned studies. To further exclude the possibility that indoleamines contributed to the fluorescence patterns observed in the study, we also labeled several ($n \approx 50$) pediveligers of *M. edulis* with antibodies raised against serotonin conjugated to bovine serum albumin with paraformaldehyde. Procedures were modified from Marois and Croll (1992) and Marois and Carew (1997). Thirty-four-day-old *M. edulis* larvae were fixed overnight in 4% paraformaldehyde in PBS. The embryos were then transferred to 70% ethanol in which they were stored at -20°C . For histology, the stored larvae were washed briefly in PBS and then placed into 10% EDTA in PBS for 20 min at room temperature. After another brief rinse in fresh PBS and overnight incubation in 4% Triton X-100 in PBS, the larvae were transferred into a 1:500 dilution of rabbit anti-serotonin antibodies (Incstar, Stillwater, MN) in PBS for 48–72 h. These latter and all subsequent steps were performed at 4°C . Larvae were then washed in PBS for 6 h before incubation overnight in 1:500 dilution of FITC-labeled goat anti-rabbit antibodies (Bio/Can Scientific, Mississauga, Ontario). Larvae were then washed in PBS and mounted in 3:1 glycerine in PBS. Specimens were viewed using an L3 filter cube (permitting 450–490 nm illumination and visualization through a 525/20 nm bandpass filter) on the Leitz microscope and were photographed using Kodak T-MAX 100 film. Specimens prepared according to these same procedures but omitting the primary antibody showed none of the fluorescence reported below.

Results

At 22 days of age, the veligers of *P. magellanicus* (about $180\ \mu\text{m}$ shell length, as measured along the major axis) possessed neither eye spots nor feet. Blue-green fluorescence, indicative of catecholamines, was located within cells in the velum (Fig. 1A) and around the mouth (Fig. 1B). Each lobe of the velum contained two to four fluorescent cell bodies, which were generally shaped as squat cones and evenly spaced in a line along the outer rim (Fig. 2A, B). Two to three small fibers, each possessing several varicosities, could be seen running between adjacent cells along each rim (Fig. 2A–C), while other fibers coursing between the most dorsal cells connected the two velar lobes. A pair of flask-shaped, fluorescent cells was located just lateral to the mouth of the veliger (Fig. 2D). A stout apical process was directed

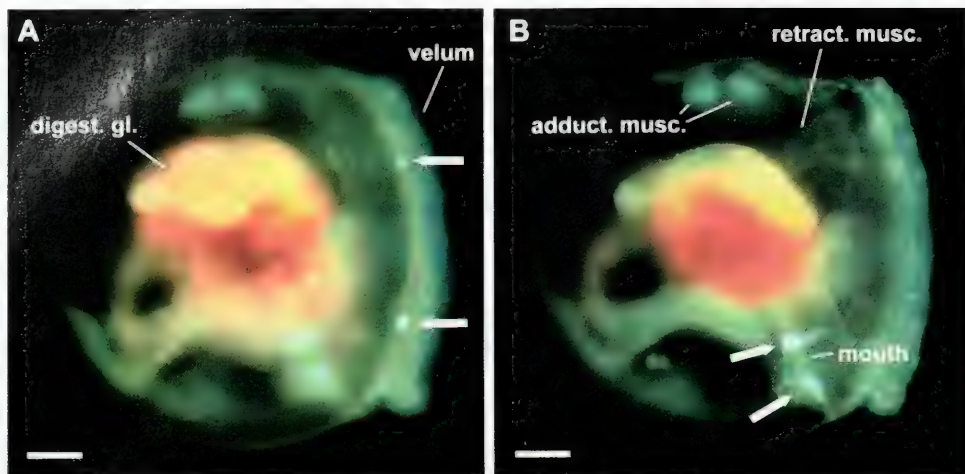


Figure 1. Wholemount, 22-day-old *Placopecten magellanicus* veliger viewed at two focal planes. (A) The partially retracted velum is to the right, and the digestive gland (digest. gl.), which exhibits red autofluorescence, lies toward the left, near the valve hinge. Aldehyde-induced, blue-green fluorescence is localized to two cells (arrows) and interconnecting fibers along the outer rim of the velum. (B) Single, blue-green fluorescent, flask-shaped cells (arrows) are also located on each side of the mouth. Velar retraction has displaced one of the cells slightly ventrally in this figure. The faint outlines of valve adductor muscles (adduct. musc.) and velar retractor muscle (retract. musc.) can also be seen. Calibration bars in A and B equal about 23 μ m.

dorsolaterally from each of these cell bodies, and thin fibers running across the midline immediately ventral to the mouth interconnected the two somata. Generally, another single fiber could be seen running between the mouth region and an area directly behind the apical tuft at the very center of the velum (Fig. 2A; see Raineri, 1995).

By 36 days of age, the larvae of *P. magellanicus* (about 275 μ m shell length) possessed prominent feet and occasionally one or two gills. The general pattern of staining in the vela of these pediveligers was essentially the same as that seen in 22-day-old larvae, although the vela were smaller in such specimens (Fig. 3C). The foot of each pediveliger contained 15–20 fluorescent cells located predominantly toward the tip and along the ventral side (sole) of the appendage (Fig. 3A–C). Distal processes from the subepithelial somata of at least some of these cells appeared to bear long cilia that projected from the surface of the foot (Fig. 3B). Axons from the fluorescent cells of the foot appeared to project centrally and converge upon the developing pedal ganglia (Fig. 3C), although such details were often partially obscured by the thicker shells and body tissues of these and older larvae. These larvae also possessed one to two additional pairs of fluorescent cell bodies near the mouth, three to four cell bodies lo-

cated within the abdominal ganglia, and a few cells along the edge of the mantle (Fig. 3C).

At 31 days, the larvae of *M. edulis* (about 255 μ m shell length) generally resembled the pediveligers of *P. magellanicus* both in general morphology and in distribution of blue-green fluorescent cells and fibers. For example, each velar lobe contained a string of three to five fluorescent cells along its outer rim (Fig. 3D). Another four to six cell bodies were clustered near the mouth, four to five cell bodies were located within the abdominal ganglia, and 25–30 cell bodies were located within the foot, often with a single isolated soma near the heel. Prominent fiber tracts connected both the cells in the foot with the pedal ganglia and the pedal ganglia with the abdominal ganglia. In addition, a few cells and fibers were occasionally detected along the edge of the mantle. (See Fig. 4A for a schematized summary of the distribution of fluorescent elements in less developed pediveliger larvae of *M. edulis*.)

At 34 days, most of the larvae of *M. edulis* (shell size remained the same as in younger specimens) exhibited signs of settling. A large percentage of the larvae crawled along the substratum using enlarged feet that were subsequently revealed to contain 30–40 fluorescent cell bodies. The vela of these larvae were smaller than those of the

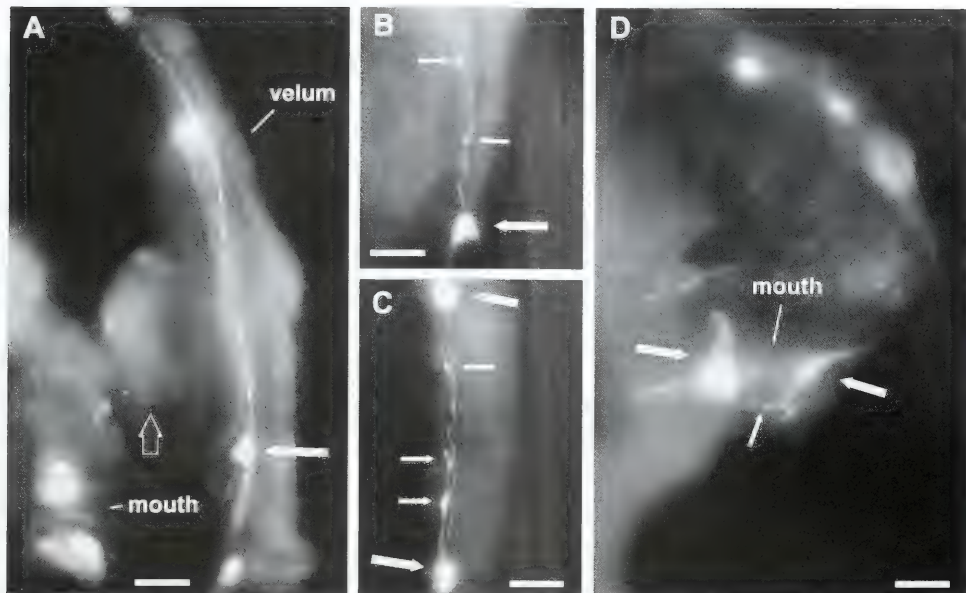


Figure 2. Aldehyde-induced fluorescence in 22-day-old veligers of *Placopecten magellanicus*. (A) Solid arrow indicates a squat, conical cell along a string of fibers on the rim of the velum. One to two fluorescent cells (out-of-focus) are located on each side of the mouth. Unfilled arrow indicates a small fiber that originates near the mouth and then terminates in what may be the apical ganglion located in the middle of the velum. (B) This shows another example of the squat conical shape of fluorescent velar cells (larger arrow). Fibers appear to exit at the base of the cell. Numerous varicosities (smaller arrows) are found along the lengths of the fibers. (C) In many cases two to three fibers with varicosities (smaller arrows) interconnect adjacent fluorescent cells (large arrows) in the velum. (D) Flask-shaped cells (larger arrows) and interconnecting fibers (smaller arrow) are found around the mouth. Calibration bars in A, B, C, and E equal about 12 μ m.

younger larvae, but strings of three to four fluorescent cells and interconnecting fibers could still be detected along each of the outer rims (Fig. 4A). Single fluorescent cells were also located at the tips of each of the two to three gill bars of these larvae, and a fiber tract could be detected connecting the most posterior gill arch with the abdominal ganglia (Fig. 3E). As with *P. magellanicus*, visualization of fluorescence in such larvae was often obscured by the shell and growing body tissues.

By 41 days, most *M. edulis* either had vela that were further reduced in size (Fig. 4B) or had completely lost their vela. Although the shell length changed little from earlier stages, new growth resulted in broader, less elliptical shells, indicating the addition of postmetamorphic dissoconch (Bayne, 1971). Prominent cells could still be seen around the mouth and within the abdominal ganglia and foot of each animal. Several of these specimens also

contained fluorescent byssal fibers running along the anterodorsal margin of the foot from its base to apex. The gills contained a total of four to five arches, each with one to two fluorescent cells. (See Fig. 4B for a schematic summary of the distribution of fluorescent elements in post-settling specimens of *M. edulis*.)

Immunocytochemical labeling of serotonin produced a completely different staining pattern than that induced by aldehydes in 34-day-old *M. edulis*. Compare Figure 5A, B (aldehyde-induced fluorescence) with Fig. 5C–F (serotonin-like immunoreactivity). The velum contained no intrinsic serotonin-like immunoreactive somata but only varicose fibers (Fig. 5C–E), which appeared to originate from three somata located near the midline just behind the center of the velum (Fig. 5E). Likewise, the foot contained no intrinsic immunoreactive somata but only varicose fibers (Fig. 5C–E), which appeared to originate

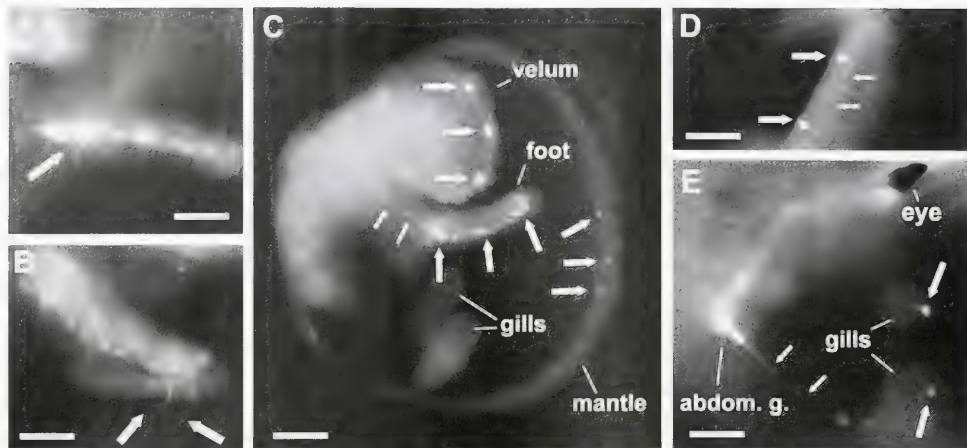


Figure 3. Aldehyde-induced fluorescence in pediveligers of *Placopecten magellanicus* and *Mytilus edulis*. (A) A number of fluorescent cells in the foot of a 36-day-old *P. magellanicus* pediveliger possess processes (arrow) that extend from subepithelial somata to the ventral surface of the foot. (B) At least some of these processes appear to bear long cilia (arrows). (C) A 36-day-old pediveliger of *P. magellanicus* with gills. Larger arrows indicate three cells in the velum, three cells in the mantle, and numerous cells in the foot. Smaller arrows indicate fluorescent fibers projecting to the pedal ganglia (not in focus). (D) Fluorescent somata (larger arrows) and fibers (smaller arrows) along the rim of the velum of a 31-day-old pediveliger of *M. edulis*. (E) A 34-day-old veliger of *M. edulis* with a prominent eyespot (eye). Larger arrows indicate cells in two gills. Smaller arrows indicate fluorescent fibers connecting gills with abdominal ganglia (abdom. g.). Calibration bars equal about 21 μm in A and B, 38 μm in C, about 30 μm in D, and about 17 μm in E.

from cells in the developing pedal ganglia. Varicose fibers innervating the mantle appeared to originate from the abdominal ganglia (Fig. 5F).

Discussion

The distribution of fluorescent cells described in this report is consistent with both chromatographic evidence (Coon and Bonar, 1986) indicating the presence of catecholamines in bivalve larvae and evidence from other studies suggesting major roles for catecholamines in such important functions as locomotion, feeding, and the triggering of settling behavior and metamorphosis (Bonar *et al.*, 1990; Beiras and Widdows, 1995a, b). However, although the presence of some catecholamine-containing cells may have been expected, the locations and relatively large numbers of neurons described in this study are surprising in light of the commonly held belief that the nervous system of molluscs is rudimentary until relatively late larval stages (Raven, 1966; Kandel *et al.*, 1981; Moor, 1983). To date, most work on molluscan neural development has focused upon embryonic cells resident within the developing central ganglia (Kempf *et al.*, 1987, 1992; Marois and Carew, 1990; Marois and Croll, 1992; Barlow and Truman, 1992).

Indeed, our study also permitted the identification of small numbers of cells in central ganglia. Aldehyde-induced fluorescence and serotonin-like immunoreactivity were present in different cell bodies and converging fibers in a region immediately behind the center of the velum, a region in which the apical or cerebral ganglion has previously been described in larval bivalves (Bayne, 1971; Raineri, 1995). Our study also identified cell bodies and fibers in the previously described developing pedal and abdominal ganglia (Bayne, 1971; Raineri, 1995). However, our work additionally demonstrates that many more catecholaminergic neurons are located peripherally and may play significant roles in the control of the velum, foot, mouth, and mantle and, in later larvae, also the gills. Furthermore, similarities between the neurons found in *P. magellanicus* and *M. edulis*, which respectively represent the Pectinidae and Mytilidae families of bivalves, suggest that the distribution of catecholamine-containing neurons may be generalized more widely across this taxon. Very similar distributions of catecholaminergic neurons have recently been found in a variety of gastropod larvae as well (E. E. Voronezhskaya, L. Hiripi, K. Elekes, and R. P. Croll, unpubl. data), thus suggesting further generalizations across molluscan classes.

The goal of the present research was to document the

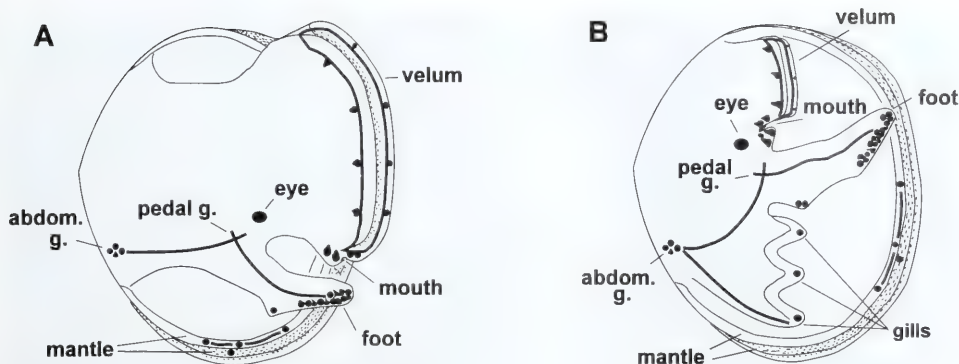


Figure 4. Distribution of aldehyde-induced fluorescent elements in *Mytilus edulis* before and after settlement. (A) Young pediveliger with eyespots (eyes). Larva contains various fluorescent cells and fibers in the velum, foot, and mantle and around the mouth. Fluorescent fibers connect the pedal ganglia (pedal g.) with cells in the foot and in the abdominal ganglia (abdom. g.). (B) After settlement the foot grows while the velum shrinks in size. Fluorescent cells are added to the developing gills, which are connected to the abdominal ganglia by means of fluorescent fibers. Lengths of major shell axes in both A and B equal about 255 μm .

presence and describe the distributions and projections of catecholamine-containing neurons in the larvae of bivalve molluscs. In the course of this work we examined specimens at the veliger and pediveliger stages of larval ontogeny and also at settlement and metamorphosis into juvenile stages. Nothing is currently known about the developmental origins of such cells, although such a study would follow one obvious avenue for future research. The present study does, however, offer unique insights into later larval life and metamorphosis. Much of the work on the neural changes associated with molluscan metamorphosis has focused upon the velum (Marois and Carew, 1990, 1997; Barlow and Truman, 1992), which had previously only been reported to receive innervation originating from the cerebral ganglia (see also Arkett *et al.*, 1987; Mackie *et al.*, 1976; but see Marois and Carew, 1997). These previous studies indicated that both the axons and originating somata disappear as the velum is lost. Our control preparations, which revealed serotonin-like immunoreactivity in premetamorphic larvae, exhibited patterns strikingly similar to those previously described in gastropods (see also Croll and Voronezhskaya, 1995; Marois and Carew, 1997); although examination of developmental changes in immunoreactivity was outside the scope of the present study, one might reasonably hypothesize that gastropods and bivalves share similarities in this regard as well. Our observations do indicate, however, that several peripheral neurons also disappear during metamorphosis. Whether the disappearance of catecholamine-containing cells in the velum is due to cell death, changes in transmit-

ter phenotype, or migration must be answered by future research.

By late larval life the foot is a complex structure used for locomotion and byssal secretion and attachment (Lane and Nott, 1975). Its motility and anterior location in the crawling pediveliger also make it ideally suited as a sensory probe. We found that the foot of the pediveliger contains relatively large numbers of catecholaminergic cells. At least some of these cells appear to have apical processes that traverse the epithelium and bear long cilia projecting from the "sole" of the foot. Similar ciliated cells have long been thought to mediate chemoreception and mechanoreception in molluscs (Croll, 1983). Furthermore, these peripherally located somata are connected to the pedal ganglia *via* fiber pathways and might therefore involve not only local control of the foot but also centrally mediated functions. Since development of the foot is acknowledged to be a necessary precondition for metamorphosis in molluscs (Bonar, 1978; see also Bayne, 1971; Cullinley, 1974), it is tempting to hypothesize that metamorphic competence results from the maturation of catecholaminergic pathways in the foot.

Consistent with our observations on larvae, both catecholamines and serotonin have long been known to be present and active in adult bivalves (Greenberg, 1960; Sweeney, 1968). More recent work confirms that such monoamines are, in fact, widespread within the tissues of bivalves such as *P. magellanicus* (Croll *et al.*, 1995; Pani and Croll, 1995). Particularly relevant to this study is the finding (Smith, 1996; S. A. Smith, J. Nason, and R. P.

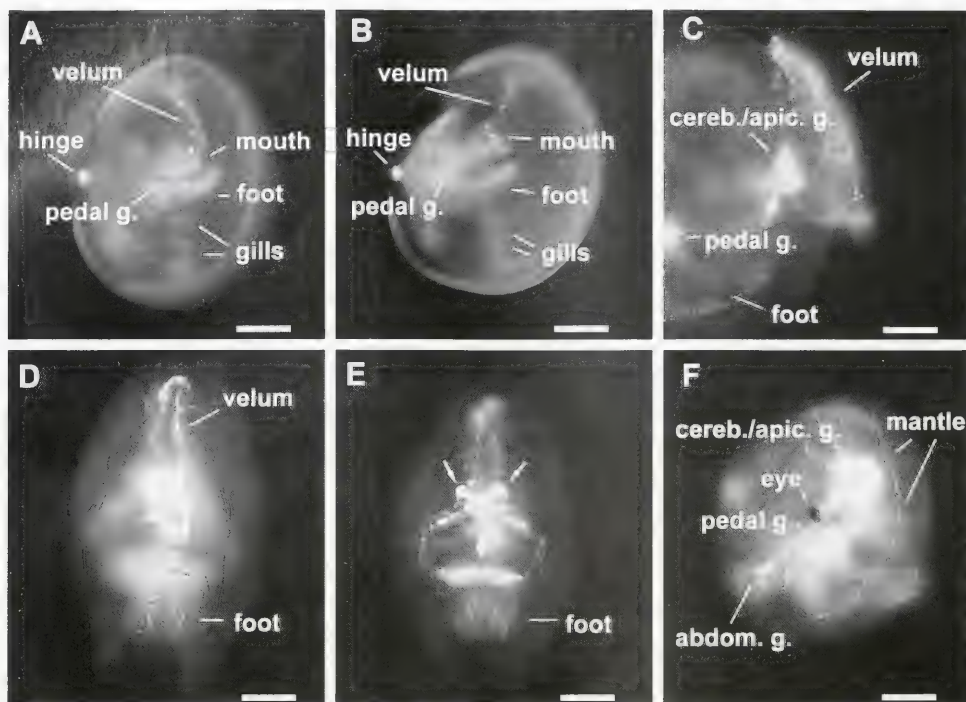


Figure 5. Aldehyde-induced fluorescence of catecholamines (A, B) and serotonin-like immunoreactivity (C-F) in pediveligers of *Mytilus edulis*. (A) Aldehyde-induced fluorescence in a 34-day-old larva with gills. A string of somata can be seen along the outer rim of the velum. Although the developing shell and body tissues often obscure details, diffuse fluorescence from numerous out-of-focus cells can be seen along the entire foot and in a concentration in the pedal ganglia (pedal g.). The hinge of *M. edulis* autofluoresces yellow whether or not glutaraldehyde is added to the fixative. (B) The velum is much smaller in this 41-day-old specimen, although aldehyde-induced fluorescence can still be seen in cells along its outer rim. Other cells can be seen in the region of the mouth and the foot. (C) Serotonin-like immunoreactivity in a 34-day-old pediveliger. Varicose fibers in the velum appear to originate from somata in the underlying cerebral or apical ganglia (cereb./apic. g.). Varicose fibers in the foot appear to originate from somata in the pedal ganglia. (D) Frontal view shows varicose serotonin-like immunoreactive fibers along the outer rim of the velum and within the foot. (E) A deeper focus shows that one medial (larger arrow) and two lateral (smaller arrows) serotonin-like immunoreactive somata in the cerebral or apical ganglia underlie the velum. (F) Lateral view shows varicose fibers running around the edge of the mantle. Immunoreactive somata or converging fibers clearly mark the locations of the developing cerebral or apical ganglia, pedal ganglia, and abdominal ganglia (abdom. g.). Calibration bars equal about 58 μm in A, B, and F and about 38 μm in C, D, and E.

Croll, unpubl. data) that adult bivalves possess catecholamine-containing neurons that reside not only within the central ganglia but also peripherally, where they are particularly concentrated in the foot and gills and surrounding the mouth, as was observed here in specimens at around the time of metamorphosis.

As the first report detailing the distribution of catecholaminergic neurons in molluscan bivalve larvae, this study

provides new foci for the study of larval form, function, and development. Previously hypothesized neuronal control mechanisms have now been tentatively identified and their actual functions can be critically tested using electrophysiological, pharmacological, and ablation techniques. These findings also suggest that larval behavior and physiology are likely to be affected by interactions between several different neurotransmitters. For example, our his-

tological work indicates that velar activity is probably influenced by both catecholamines and serotonin, as suggested by previous pharmacological studies (Beiras and Widdows, 1995a). The presence of acetylcholinesterase activity suggests that acetylcholine might also play important roles in larval function (Raineri, 1995). The finding of Phe-Met-Arg-Phe-NH₂ (FMRamide)-like immunoreactivity in early larvae of gastropods (Croll and Voronezhskaya, 1995, 1996) and bivalves (R. P. Croll and E. E. Voronezhskaya, unpubl.) adds yet another layer of complexity to our view of neural functions in molluscan larvae. It is likely that the catalog of probable neuroactive substances will grow as specific labels are increasingly applied to the study of such larvae, and further elucidation of the neural pathways in bivalve larvae will provide a better understanding of the coordination and control of larval locomotion and feeding, and the complex processes of settlement and metamorphosis.

Acknowledgments

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Nuclear Microprobe Mapping of Statoliths of Chokka Squid *Loligo vulgaris reynaudii* d'Orbigny, 1845

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Abstract. Loliginid squid statoliths were broken in half and their elemental composition was assessed using the nuclear microprobe technique. Proton induced X-ray emission and proton backscattering were used simultaneously. True, quantitative maps of calcium and strontium distribution in the frontal plane were obtained using a rapid-matrix-transform method called Dynamic Analysis. These measurements were complemented by observations with scanning electron microscopy and light microscopy. In juvenile and adult statoliths, Sr was concentrated in the wing and adjacent areas, whereas the Ca concentration was highest on the edge of the lateral dome. In contrast, Sr and Ca were uniformly distributed in the statoliths of paralarval squid. Increments showed best definition in the areas anterior and adjacent to the wing, corresponding to areas of high Sr content. Although temperature, sex, maturity, and the administration of oxytetracycline may influence the quantitative distribution of Ca and Sr in the statoliths of adult squid, they do not appear to affect the general pattern described above. The finding that Sr is concentrated in regions adjacent to the macula where the clearest increments are found in loliginid statoliths supports the hypothesis linking strontium with the regulation of statolith deposition and the definition of daily increments.

Introduction

Considerable interest has been directed at the use of statoliths to estimate the age of squid. However, as with determining the age of fish from their otoliths, several unresolved problems complicate the estimates derived from the analysis of statolith microstructure. At present, very subjective criteria are applied to distinguish daily increments from other potentially confusing microstructural features such as sub-daily rings and checks. This element of subjectivity is probably the primary cause of the highly variable age estimates reported for some species of squid (Villanueva, 1992, vs. Lipinski *et al.*, 1993; Natsukari *et al.*, 1993, vs. Arkhipkin *et al.*, 1996). The interpretation of such features has been simplified through an understanding of the physiology of their underlying causal mechanisms (Gauldie *et al.*, 1995). In their paper on modeling of otolith growth, Romanek and Gauldie (1996) stated: "Theoretical models of otolith growth would greatly assist in establishing criteria for the validation of age marks, but they are surprisingly uncommon given the extent of disagreement between age estimation techniques." Research towards this goal is relatively well advanced for fish otoliths (Gauldie and Nelson, 1990; Gauldie *et al.*, 1995; Romanek and Gauldie, 1996), but apart from the studies of Morris (1988) and Lipinski (1986, 1993), very little attention has been directed to the physiological mechanisms by which squid form statoliths.

We feel that there are not enough basic data on the elemental composition and dynamics of various components of the vestibular systems of fish and squid (e.g.,

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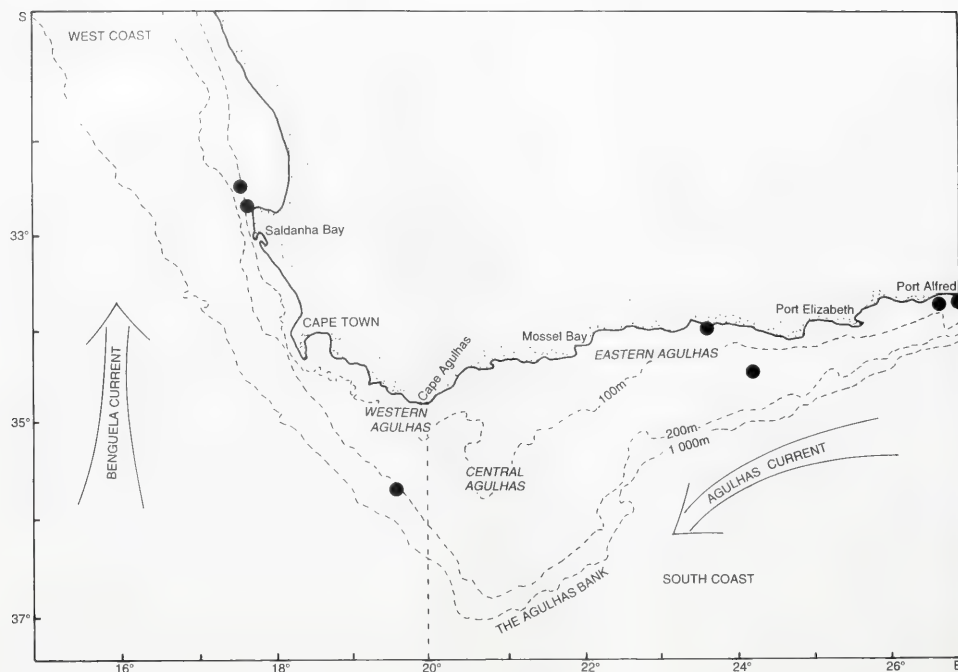


Figure 1. Distribution of squid sampling sites.

otoliths/statoliths, endolymph fluid, maculae) to improve the existing models. Data on statolith deposition are particularly important, as emphasized by Romanek and Gauldie (1996). An understanding of this process could provide valuable insights into the nature and operation of the circadian rhythm presumed to be implicit in the deposition of daily increments, as well as clarifying the structural and functional characteristics of the increments. This latter aspect is felt to be of crucial importance in the application of the statolith-based aging technique. Our understanding of these characteristics in fish otoliths has been greatly furthered by investigations of the elemental composition of otoliths. For example, Gauldie *et al.* (1992, 1995) used a proton microprobe to evaluate otolith age estimates of two fish species by comparing patterns of checks, cycles in increment width, and cycles in strontium and calcium composition. Mugiya and Satoh (1995) were able to analyze calcium and strontium levels within increments by using X-ray microanalysis. Unfortunately, the goal of the latter study cannot be realized in loliginid squid because the increment widths in the statoliths are

typically on the order of 1 μm or less, below the resolution limits of current microanalytical techniques. However, as has been demonstrated by Gauldie *et al.* (1992, 1995), microanalysis of elemental composition on a larger scale than that of increments can generate valuable information about the deposition of otoliths and statoliths, and thus may help answer questions about the formation of increments.

In the current work we sought (1) to detect any qualitative associations between statolith microstructure (specifically increment appearance) and the distribution of calcium and strontium within squid statoliths, and (2) to assess whether any patterns discovered were consistent between sexes, stages of development, and ambient temperatures. We also considered the influence of oxytetracycline (OTC) marking on the elemental composition of statoliths. Results of validation experiments employing this substance as a chemical marker indicated that its incorporation into the statoliths may have been associated with some physiological stress, and hence may have had some influence on the crystals deposited while the

Table I

Description of material used in the PIXE analysis

Type of material	n	ML (mm)	Capture date	Location	Sex and maturity
paralarvae	4	<2	10/10/91	Kromme Bay	
juveniles	2	65	15/09/92	Plettenberg Bay	M I
		78	15/09/92	Plettenberg Bay	M I
adults (mixed material)	6	154	15/09/92	Plettenberg Bay	M III
		175	11/11/93	Kromme Bay	F III
		198	16/09/92	S. of TCNP	M III
		250	05/01/94	Agulhas Bank	M II
		263	12/11/93	Kromme Bay	M V
		350	11/11/93	Kromme Bay	M V
adults (OTC marked)	5	171	01/03/93	aquarium†	F III
		182	14/11/93	aquarium†	M IV
		200	01/03/93	aquarium†	M III
		240	22/12/93	aquarium†	M V
		324	15/11/93	Kromme Bay	M V
adults (warm water)	3	182	20/09/92	Port Alfred	F V
		214	20/09/92	Port Alfred	M III
		268	20/09/92	Port Alfred	M V
adults (cold water)	3	179	13/01/94	St. Helena Bay	F II
		202	13/01/94	St. Helena Bay	M II
		225	13/01/94	St. Helena Bay	M III

† M—male, F—female; maturity stages (I–VI) assessed according to the scale of Lipinski and Underhill (1995).

‡ Aquarium maintenance material was all captured in False Bay.

OTC was being metabolized (Lipinski *et al.*, unpub. data).

Strontium concentrations in biogenic calcium carbonates are often a limiting factor in studies of this nature. The proton microprobe system is able to measure elements at levels approaching a few parts per million, with the further advantage of a data acquisition system that permits quantitative mapping with full-spectrum processing at every pixel in the image.

Materials and Methods

Statolith collection and preparation

Statoliths of chokka squid (*Loligo vulgaris reynaudii*) were collected during several summer cruises of RS *Africana*, RS *Algoa*, and various commercial vessels in the years 1992–1994. The area of collection (Fig. 1) extended from St. Helena Bay on the west coast of South Africa to Port Alfred on the southeast coast. The list of collected material is given in Table I. The three warm-water squid were collected at 30-m depths at sites near Port Alfred

influenced by the warm Agulhas Current. The bottom temperature at these sites was 17.6°C at the time of collection. In contrast, the three cold-water squid were collected in cold upwelled water near St. Helena Bay at depths of 100 m; the bottom temperature at these sites was 9.1°C at the time of collection. The OTC-marked statoliths were obtained from laboratory validation studies in which up to 0.1 ml of an OTC solution (Lipinski, 1986) was injected into the ventral mantle musculature of squid to mark the statoliths with a fluorescent label. After marking, the squid were maintained for periods ranging from 2 to 30 days in aquaria supplied with a continuous flow of fresh seawater and exposed to the ambient light regime. The squid were fed a diet of live fish *ad libitum* for the duration of the experiments.

Statoliths were dissected according to the method of

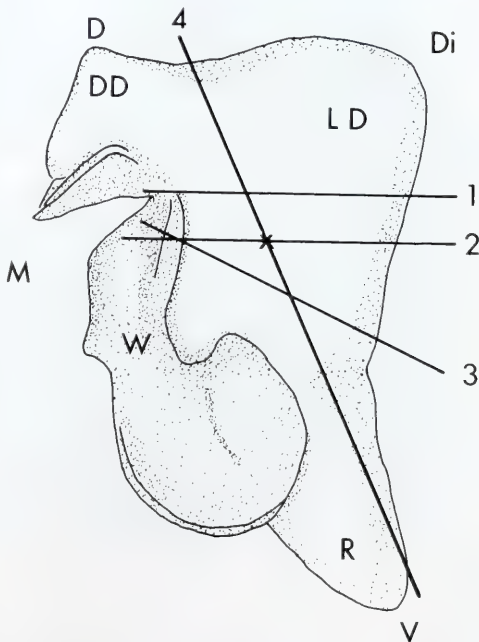


Figure 2. Diagram illustrating an anterior view of the statolith from the left statocyst of a chokka squid, showing the orientation and parts of squid statoliths, as well as the break and section planes used in this study. D—dorsal, V—ventral, M—medial, Di—distal (lateral), DD—dorsal dome, LD—lateral dome, R—rostrum, W—wing, 1—frontal break above the nucleus (the position of the nucleus is indicated with an 'x'), 2—frontal break through the nucleus, 3—oblique frontal break below the nucleus, 4—sagittal section through the nucleus.

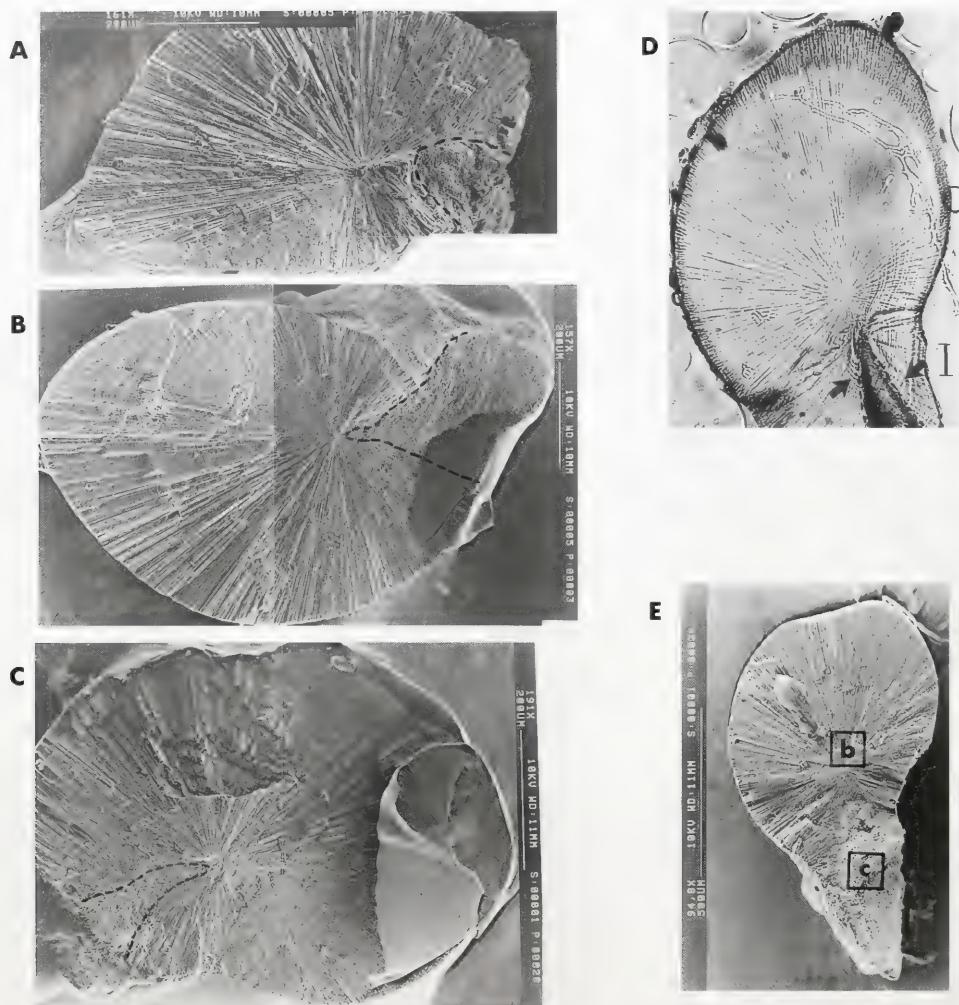


Figure 3. Internal structure of chokka statoliths; the inclusion of wing crystals is outlined with a dotted line. (A) Scanning electron micrograph (SEM) of statolith (ML = 175 mm; ♀ V) broken below the nucleus (plane 3 in Fig. 2). (B) SEM of statolith (ML = 268 mm; ♂ V) broken at the level of the nucleus (plane 2 in Fig. 2). (C) SEM of statolith (ML = 179 mm; ♀ II) broken above the nucleus (plane 1 in Fig. 2). (D) Light micrograph of ground and polished frontal section of a chokka statolith (ML = 57 mm; juvenile) showing the wing and position of clearest increments (arrows); the scale bar is 50 μ m. (E) SEM of statolith (ML = 324 mm; ♂ V) illustrating the position of the microprobe maps presented in Fig. 6B, C.

Lipinski (1981) and stored dry in small plastic tubes. They were fractured in the frontal plane using a clean and sterilized scalpel blade. The line of the break was

determined by the approximate position of the nucleus and varied relatively little (Fig. 2). Ten statoliths, however, were broken below the nucleus where the rostrum

forms an angle with the lateral dome (plane 3 in Fig. 2), and ten statoliths were broken above the nucleus (plane 1 in Fig. 2). The surface of the break was then viewed under a stereomicroscope, and only statoliths with relatively clean and even surfaces were accepted for further preparation. The statolith halves were mounted on a Formvar membrane spread on a thin aluminum frame using Araldite Rapid glue. A second frame with Formvar was glued on top of the statolith's frontal surface and coated with a thin layer of carbon. Once the nuclear microprobe observations had been completed, the samples were re-mounted on aluminum stubs, re-coated with carbon, and viewed under a Stereoscan 200 scanning electron microscope (SEM). To locate areas of the most prominent increments, statoliths were prepared according to Lipinski and Durholtz (1994) and viewed and photographed under a Zeiss light microscope. Terminology used is after Clarke (1978) and Lipinski *et al.* (1991).

Microprobe measurements

The selection of the microanalytical technique for this study was based on two criteria, namely lateral resolution and sensitivity. Ideally, a technique with a lateral resolution of about $0.5\ \mu\text{m}$ or less (the spatial scale of increments in squid statoliths), with a sensitivity permitting quantitative mapping of the elements of interest, is required. Since Sr concentration was of particular interest in this study, the analytical technique had to be capable of measuring this element at levels approaching a few parts per million. Although the proton microprobe is capable of this level of sensitivity, it cannot fulfill the criterion for lateral resolution. Techniques that provide such high levels of resolution (such as electron microprobes in energy dispersed spectrometry mode, or electron energy loss spectroscopy) require very thin samples; these are almost impossible to obtain from squid statoliths, which tend to disintegrate when very fine sectioning is attempted. Furthermore, it is doubtful whether such techniques are suitably sensitive for Sr analysis. To our knowledge, no technique currently satisfies both the lateral resolution and sensitivity requirements of this study. The use of the proton microprobe consequently represents a compromise between these two criteria.

The analyses were performed with 3.0 MeV protons, using the nuclear microprobe of the National Accelerator Centre (NAC). The details of the experimental setup have been reported previously (Tapper *et al.*, 1993; Prozesky *et al.*, 1995). Two complementary techniques, proton induced X-ray emission (PIXE) and proton back scattering (BS) were used simultaneously. The beam spot size was about $3\ \mu\text{m} \times 3\ \mu\text{m}$ for proton currents of 200–400 pA. The proton currents were maintained within this range



Figure 4. Light micrograph of a ground and polished sagittal section of a statolith (ML = 320 mm; δV) showing the transverse axis of crystallization (arrows). The scale bar is $100\ \mu\text{m}$.

because higher currents destroyed the Formvar layer mounted on top of the statolith's frontal surface, affecting the charge collection.

PIXE spectra were registered using a Si(Li) X-ray detector, situated about 37 mm away from the target at 135° to the incoming beam direction and separated from the evacuated specimen chamber by means of an intervening

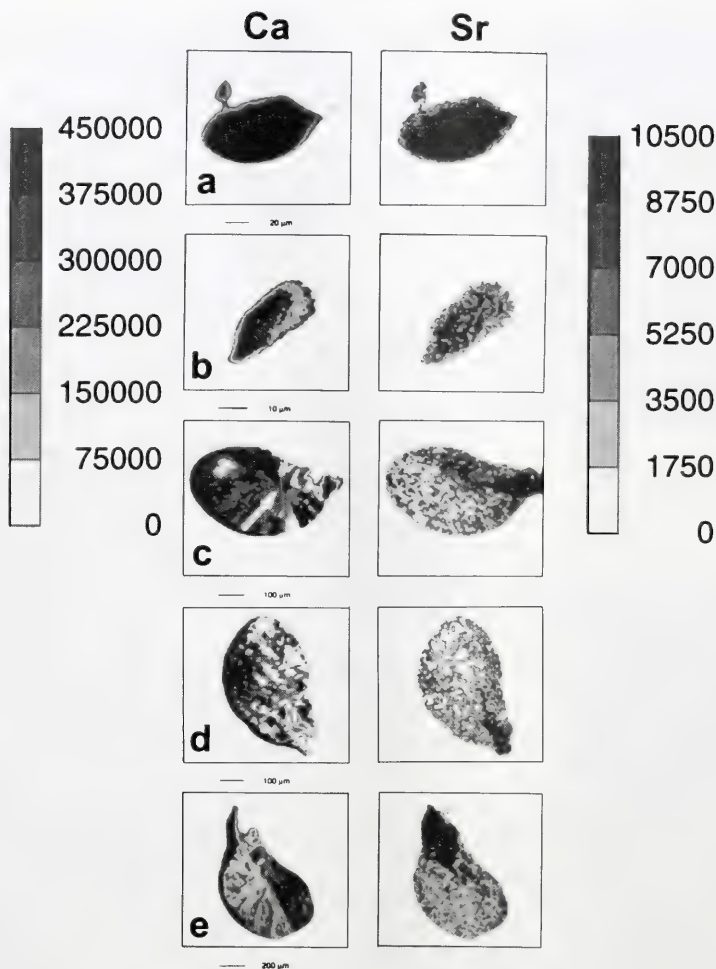


Figure 5. Ontogenetic changes in calcium and strontium concentrations in the squid statolith (values in ppm): (a) paralarva; (b) paralarva; (c) ML = 65 mm, ♂ I; (d) ML = 78 mm, ♂ I; (e) ML = 214 mm, ♂ III.

25- μ m Mylar window. An additional 40- μ m Al absorber was used to reduce the intensity of Ca K X-ray lines and allowed the simultaneous measurements of Ca as well as of minor and trace elements. The setup was calibrated using thick pure elements and glass standards as well as thin standards from Micromatter (Prozesky *et al.*, 1995; van Achterbergh *et al.*, 1995). PIXE spectra were ana-

lyzed using the GeoPIXE suite of programs (Ryan *et al.*, 1990; Ryan and Jamieson, 1993). This package allows the analysis of X-ray spectra with complete corrections for beam stopping, X-ray attenuation, and secondary fluorescence of thick targets.

Backscattered protons were registered with an annular Si surface barrier detector, situated at 176° to the incom-

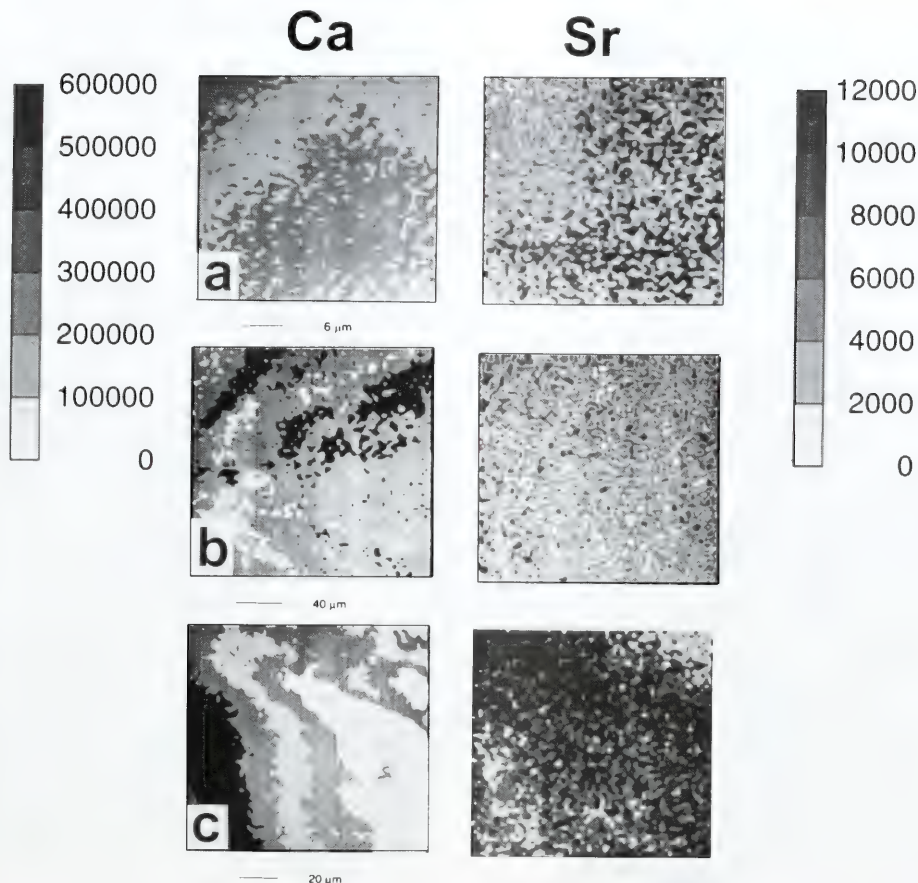


Figure 6. Ontogenetic and structural changes in calcium and strontium concentrations (values in ppm) in selected structures of squid statoliths: (a) paralarva, nucleus area; (b) adult (ML = 324 mm, ♂ V), nucleus area (see Fig. 3E); (c) adult (same individual as b, see Fig. 3E), wing area.

ing beam direction. The simulations of proton BS spectra were performed with a modified version of RUMP, a software package that performs the simulations of Rutherford backscattering spectra (Doolittle, 1986), using experimental cross-sections for isotopically natural C and O at a laboratory angle of 170° (Amirikas *et al.*, 1993). No correction was made for this slight difference in angle between the experimental and simulated geometry.

Elemental maps were made using a recently developed rapid matrix transform method called Dynamic Analysis

(DA) (Ryan and Jamieson, 1993; Ryan *et al.*, 1995). This is a part of the GeoPIXE package and allows for the production of true elemental images. The images are inherently overlap-resolved and background-subtracted, and the maps are generated on-line. Final maps give quantitative elemental images and the intensity is given in parts per million.

Mapping was performed systematically. First one or more point analyses were made in selected regions of the statolith to find a representative PIXE spectrum with all

Table II

Calcium and strontium composition of squid statoliths

Statolith	Element	Concentration (ppm)		Sr/Ca (%)
		mean	SD	
paralarvae	Ca	3.763×10^3	5.304×10^3	2.25
	Sr	8.451×10^1	157	
	Ca	3.374×10^3	4.779×10^3	2.34
	Sr	7.892×10^1	164	
	Ca	3.266×10^3	4.550×10^3	3.03
	Sr	9.906×10^1	231	
ML 324 mm (OTC*) (lateral dome)	Ca	3.468×10^3	4.894×10^3	2.20
	Sr	7.614×10^1	143	
ML 324 mm (OTC*) (wing)	Ca	4.415×10^3	6.230×10^3	1.88
	Sr	8.321×10^1	149	
	Ca	3.461×10^3	4.789×10^3	3.39
	Sr	1.174×10^2	206	

* Statoliths that were labeled with oxytetracycline.

elements present and to evaluate the specimen thickness for proper matrix corrections. It was found that the specimens were infinitely thick for microanalytical purposes (*i.e.*, their thickness exceeded the depth of penetration of the proton beam), and approximation of the sample composition as CaCO_3 was sufficient for matrix correction. The influence of the Formvar layer on top of specimens (about $0.5 \mu\text{m}$ thick) was found to be negligible, and the correction for this layer was not included.

Scan size was selected according to the size of individual specimens. Scanned regions were divided into 64×64 pixels. The Interactive Data Language (IDL) software package (Research Systems, Inc., 1993) was used for data presentation, and the maps are contours linking pixels with similar values.

Statistical analysis of the influence of temperature

As a preliminary investigation of the possible influence of temperature on calcium and strontium levels in squid statoliths, the data from three individuals of similar size and maturity from each of the two temperature regimes were compared. Raw data files obtained from the microprobe analyses provided measurements of calcium and strontium concentration within each pixel of the maps. To ensure that the data were representative of the temperature regime at which the squid were captured, analysis was restricted to points around the periphery of the lateral dome (*i.e.*, the most recently deposited mineral). Concentrations of both calcium and strontium were obtained for each pixel around the periphery, Sr/Ca ratios were calcu-

lated for each pixel, and the data were subjected to an analysis of variance (ANOVA) to test for differences between the statoliths and between the temperature regimes. STATISTICA, a commercially available software package, was used for all statistical procedures.

Results

Morphology of the surface of the statolith frontal break

SEM observations of the surfaces of the frontal breaks (indicated in Fig. 2) showed aragonite crystals radiating outwards from a central point (Fig. 3). In the breaks through the nucleus (plane 2 in Fig. 2), this central point is clearly the focus. However, in breaks above and below the nucleus (planes 1 and 3 in Fig. 2), the central point is not the focus, suggesting that a previously undescribed axis of crystallization extends dorsoventrally from the focus. LM observations of ground and polished sagittal sections (plane 4 in Fig. 2) through the focus clearly show the axis extending ventrally from the focus beyond the nucleus and possibly the protostatolith, into the rostrum (Fig. 4). The dorsal component of the axis above the focus is not clearly apparent, and probably does not extend very far, if at all, beyond the nucleus.

The breaks differed, however, with respect to the medial inclusion of the irregularly orientated wing crystals into the lateral dome. In the planes above and below the focus, the inclusion of wing crystals does not reach the axis of crystallization (Fig. 3A, C). In contrast, when a statolith is broken through the focus, the triangular wing inclusion clearly originates at the focus. LM observations of the frontal break in the same plane as the nucleus showed that the clearest increments were located in the regions adjacent and anterior to the triangular inclusion of wing crystals (Fig. 3D).

Maps of Ca and Sr distribution in loliginid statoliths: ontogenetic changes

In chokka hatchlings, Ca and Sr seem to concentrate in the middle of the statolith, but this may be an artifact related to the thickness of the sample (see Materials and Methods). It is more likely that these elements are distributed uniformly through the break. Sr levels were considerably higher than those measured in the lateral domes of older squid, but were either comparable to levels in the wings of both juvenile and adult squid, or intermediate (Figs. 5 and 6; Table II).

In juveniles and adults of mantle lengths 65 mm and larger, patterns of Ca and Sr were generally very similar among individuals. The distribution of Sr showed the

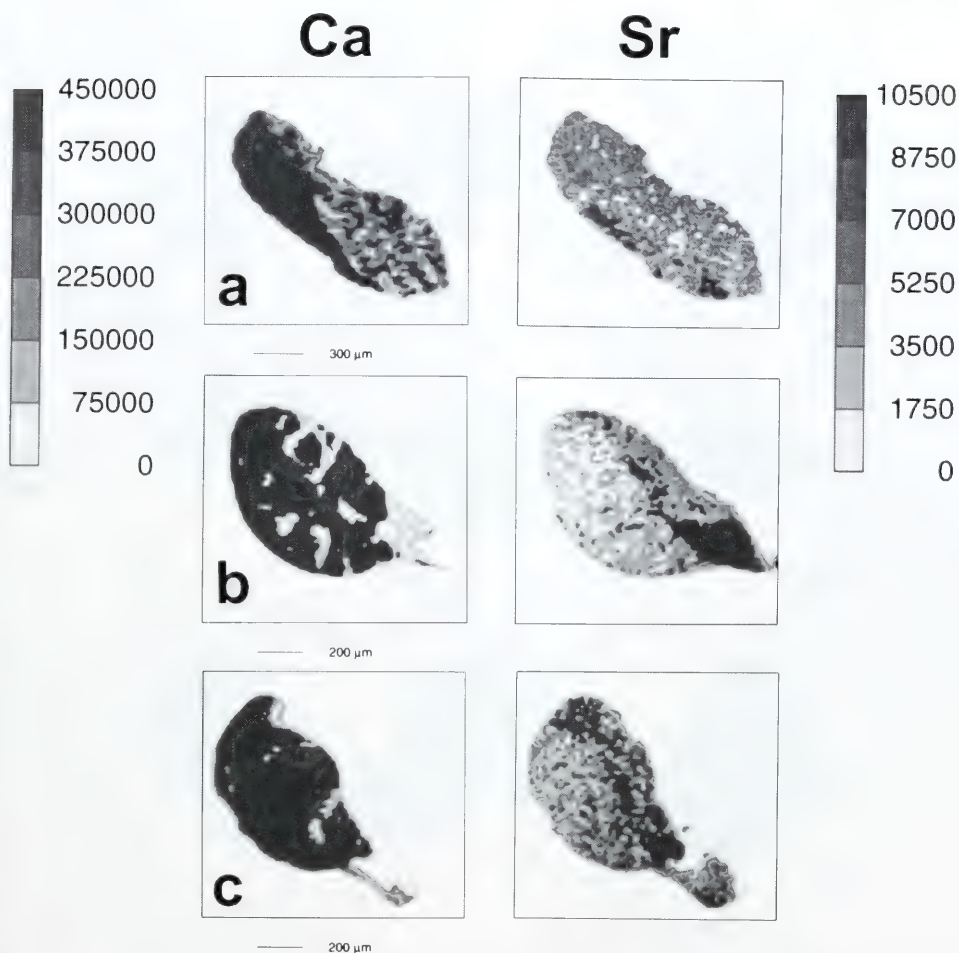


Figure 7. Calcium and strontium concentrations (values in ppm) in transverse sections of the squid statolith at levels: (a) below the nucleus (ML = 175 mm, ♀V); (b) at the nucleus (ML = 182 mm, ♂IV, OTC marked squid, 19 days post-marking); (c) above the nucleus (ML = 179 mm, ♀II).

most striking and consistent pattern. In all planes investigated, Sr exhibited uniformly high concentrations in the wing crystals (Figs. 5, 7, 9) and adjacent areas, with patches of high Sr concentration extending along a curving axis into the anterior regions of the lateral dome. Ca distribution patterns were less clearly defined, showing great variability among individuals. In general however, Ca concentrations were higher in the lateral domes (par-

ticularly in the peripheral regions) and lower in the wing area (Fig. 5, 7, 8).

Effects of temperature, sex, and oxytetracycline injection

Although the maps of statoliths collected in warm and cold water showed a great deal of variability, the general

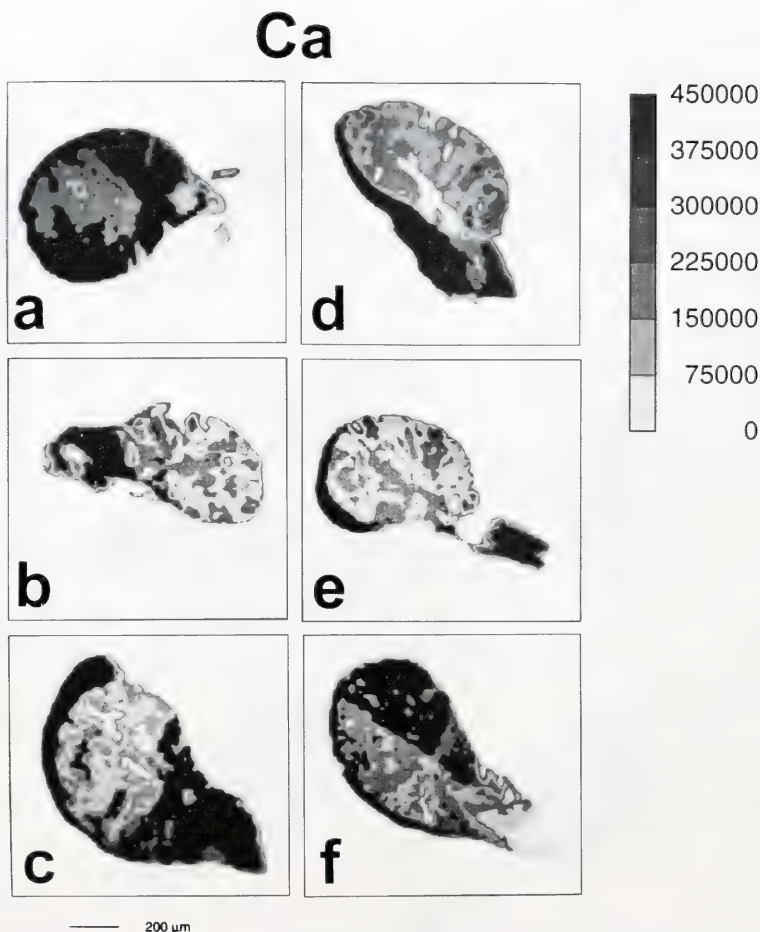


Figure 8. Calcium concentrations (values in ppm) in statoliths from cold waters (a–c) and warm waters (d–f). (a) ML = 179, ♀ II; (b) ML = 202 mm, ♂ II; (c) ML = 225 mm, ♂ III; (d) ML = 182 mm, ♀ V; (e) ML = 214 mm, ♂ III; (f) ML = 268 mm, ♂ V.

structural patterns described above did not appear to differ between the two temperature regimes (Figs. 8 and 9). Whereas mean calcium concentrations around the periphery of the lateral domes were significantly different between the two temperatures (Table III), with mean calcium concentrations slightly higher in cold-water statoliths than in warm-water statoliths, no difference was found in mean strontium concentrations. Corresponding Sr/Ca ratios showed significant differences with tempera-

ture: ratios were higher in warm-water statoliths than in those from cold waters (Table III). Although the variation in calcium concentrations and Sr/Ca ratios between the two temperature groups was statistically significant, variation in these variables, as well as in the strontium concentrations, was found to be significant among the statoliths within each temperature group (Table IV). *A posteriori* Tukey multiple comparison tests indicated that the differences in calcium, strontium, and Sr/Ca levels among the

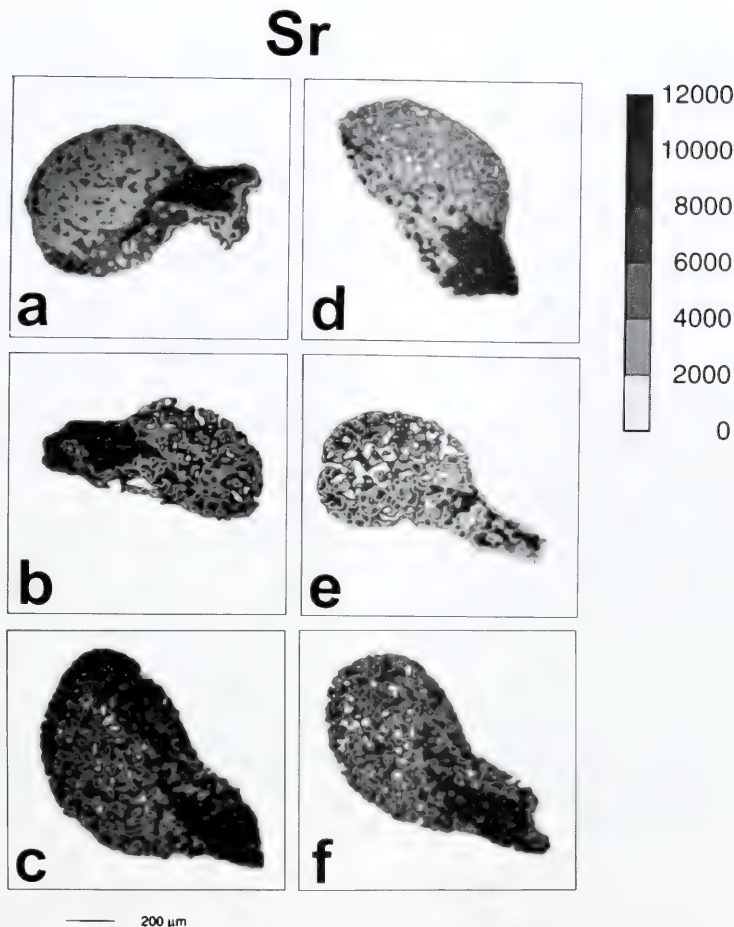


Figure 9. Strontium concentrations (values in ppm) in statoliths from cold waters (a–c) and warm waters (d–f). Same individuals as in Fig. 8.

various statoliths had no clear pattern that could possibly be attributed to a temperature effect (Table V).

No consistent sex- and maturity-related differences were detected (Figs. 7 and 8). OTC incorporation into the statolith had no apparent effect on the structural patterns observed (Fig. 7B vs. Figs. 8, 9).

Other elements present

In addition to Ca and Sr, the elements detected by PIXE were Cr, Fe, Zn, Cu, and Br. Cr and Fe, which

were confined to the extreme borders of the specimens, with very low concentrations within the body of the statolith, were judged to be experimental artifacts introduced during the preparation process rather than genuine components of the statoliths. The remaining elements (Zn, Cu, and Br) were present in only some of the statoliths (Figs. 10 and 11), but when present, their distribution suggested that they were not impurities. The distribution of these elements cannot be deduced from these limited data, but a more detailed discussion of the distri-

Table III

Comparisons of mean calcium concentrations (ppm), mean strontium concentrations (ppm) and Sr/Ca ratios between warm- and cold-water statoliths

Statolith type	Calcium				Strontium				Sr/Ca			
	Mean	SD	n	ANOVA*	Mean	SD	n	ANOVA*	Mean	SD	n	ANOVA*
Warm-water	247,378	52,606	394	$F = 76.497$	5210	1212	394	$F = 3.779$	0.0221	0.0075	394	$F = 14.114$
Cold-water	281,161	57,518	419	$P < 0.001$	5370	1131	419	$P = 0.052$	0.0201	0.0074	419	$P < 0.001$

* ANOVA conducted on data pooled over all statoliths in each temperature group.

bution of these elements can be found in Lipinski *et al.*, (in press).

Discussion

Morphology

Interpretation of previously reported data on statolith structure (Lipinski *et al.*, 1991; Lipinski, 1991, 1993) suggests a single nucleation site, with calcification radiating from that site. We propose an alternative, namely that further calcification proceeds along an axis extending dorsoventrally from the focus. Further research should be directed at this axis—specifically at the potential complications in interpreting increment patterns and increment counts.

Maps: ontogenetic changes of Ca and Sr content

The ontogenetic changes we have described in Ca and Sr distribution patterns in the statoliths of *Loligo vulgaris reynaudii* provide information concerning the deposition of squid statoliths. We interpret the patterns observed in

paralarval statoliths as showing uniformly high concentrations of Ca and Sr. With continuing growth of the animal, and deposition of statolith layers, there is a shift to increasing Ca concentration in peripheral areas of the statolith (especially posterior), distant from the wing area. Sr, on the other hand, shows consistently high concentrations in the wing crystals themselves, in areas adjacent and anterior to the wing, and in the anterior regions of the lateral dome.

These observations are consistent with the hypothesis of biomineralization in the squid statocyst proposed by Lipinski (1993), namely that Sr is secreted into the endolymph from the cells of the macula, whereas Ca is supplied from sites distant to the macula as suggested by Morris (1988). With the onset of deposition in squid embryos, Sr precipitation occurs at the macula (reactions according to Lipinski, 1993), accounting for the high Sr concentrations in paralarval statoliths. As deposition progresses and the statolith increases in size, deposition sites on the posterior and lateral areas of the statolith become increasingly distant from the Sr supply in the macula, hence Sr concentrations are low in these areas.

Table IV

Comparisons of mean calcium concentrations (ppm), mean strontium concentrations (ppm) and Sr/Ca ratios between warm- and cold-water statoliths

Statolith*	Calcium				Strontium				Sr/Ca			
	Mean	SD	n	ANOVA	Mean	SD	n	ANOVA	Mean	SD	n	ANOVA
Warm-water												
W1 (268 mm)	267,247	52,294	123		5207	1337	123		0.0200	0.0059	123	
W2 (182 mm)	229,668	63,690	138	$F = 17.916$	5430	1277	138	$F = 4.286$	0.0256	0.0098	138	$F = 25.817$
W3 (214 mm)	247,361	33,452	158	$P < 0.001$	5020	1011	158	$P = 0.014$	0.0206	0.0047	158	$P < 0.001$
Cold-water												
C1 (202 mm)	282,303	31,393	128		5023	858	128		0.0179	0.0032	128	
C2 (179 mm)	292,511	46,495	139	$F = 6.441$	5396	957	139	$F = 11.854$	0.0189	0.0044	139	$F = 25.381$
C3 (225 mm)	267,588	81,416	127	$P < 0.001$	5693	1420	127	$P < 0.001$	0.0237	0.0109	127	$P < 0.001$

* Mantle length shown in parentheses.

Table V

Results of Tukey multiple comparison tests on calcium and strontium data for warm- and cold-water statoliths

Statolith	Calcium					Strontium					Sr/Ca				
	C 2	C 3	W 1	W 2	W 3	C 2	C 3	W 1	W 2	W 3	C 2	C 3	W 1	W 2	W 3
Cold-water															
C 1	0.650	0.244	0.237	0.000	0.000	0.102	0.000	0.810	0.054	1.000	0.893	0.000	0.180	0.000	0.028
C 2		0.003	0.003	0.000	0.000		0.313	0.798	0.999	0.073		0.000	0.793	0.000	0.309
C 3			1.000	0.000	0.032			0.013	0.458	0.000			0.001	0.253	0.006
Warm-water															
W 1				0.000	0.043				0.656	0.799				0.000	0.987
W 2					0.068					0.037					0.000

Note: Results significant at the 5% level are indicated in bold.

The dorsoventral axis of crystallization described earlier may account for the curving axis of higher Sr concentrations that extends into the anterior regions of the lateral dome. Since Ca supply sites are distant from the macula, Ca availability does not become limited as the statolith increases in size, hence the Ca levels are higher in the peripheral area of the lateral dome.

The relevance of these deductions to incremental growth of squid statoliths rests in the importance of strontium. Lipinski (1993, p. 259) suggested that good increment visibility is associated with high Sr concentration in squid statoliths. The Sr distribution patterns described here confirm that concept. The area adjacent and anterior to the cone-shaped intrusion of wing crystals shows the greatest promise for increment enumeration studies, since it is in this region that increments are consistently visible to the margin (Lipinski *et al.*, unpub. data; and see Fig. 3D). This region is the area of the adult statolith that is closest to the cells of the macula, and hence the proposed Sr supply, and shows high Sr contents in all microprobe maps of adult statoliths. Clear increments also occur along a curved axis extending from the nucleus into the anterior regions of the lateral dome; again, this distribution corresponds to the areas of high Sr concentration observed in this study. In contrast, marginal areas of the lateral dome of adult statoliths generally show no increments; these regions correspond to areas of low Sr concentration.

The relationship between Sr distribution patterns and increment visibility can be explained by the results of Mugiya and Satoh (1995), who documented relatively higher Sr concentrations in the discontinuous zones of increments in fish otoliths. Each increment comprises a "light" incremental zone and a "dark" discontinuous zone. Clear increments are a product of clearly defined discontinuous zones, and hence layers of high Sr content.

We can only speculate on the physiological processes

linking Sr content to statolith growth, but Hanlon *et al.* (1989) have clearly demonstrated that Sr availability is critical to normal statolith growth. In a number of cephalopod species, a lack of Sr in ambient seawater resulted in either a complete absence of statoliths or the formation of grossly deformed statoliths. Mugiya and Tanaka (1995) indicated that in fish otoliths, strontium may successfully compete with calcium in binding to some kinds of protein. We suggest that a Sr-protein complex stabilizes the aragonite wing crystals in the reverse reactions of deposition and resorption, and that these crystals then serve as a template for the subsequent deposition of other parts of the statolith. We therefore argue that statolith formation is a process triggered, and possibly controlled, by Sr levels in the statocyst that are linked with the protein component. Details of this process, as well as the extent to which it is coupled or modified by other factors (such as pH; Morris, 1988), need to be investigated.

Effects of temperature, sex, and oxytetracycline injection

Statoliths from individuals collected more than 600 km apart in cold (9°C) and warm (18°C) waters showed no major differences in Ca and Sr distribution patterns, indicating that temperature does not influence the basic biomineralization processes occurring in squid statocysts. Sex and maturity also appeared to have no influence on the basic distribution patterns of Sr and Ca. The between-individual variability in Ca and Sr concentrations observed in this study indicates that a larger sample size is required to establish whether temperature, sex, and maturity influence the concentrations of these elements in statoliths.

The data obtained from the 5 OTC-marked statoliths indicated that the incorporation of this compound did not

Zn

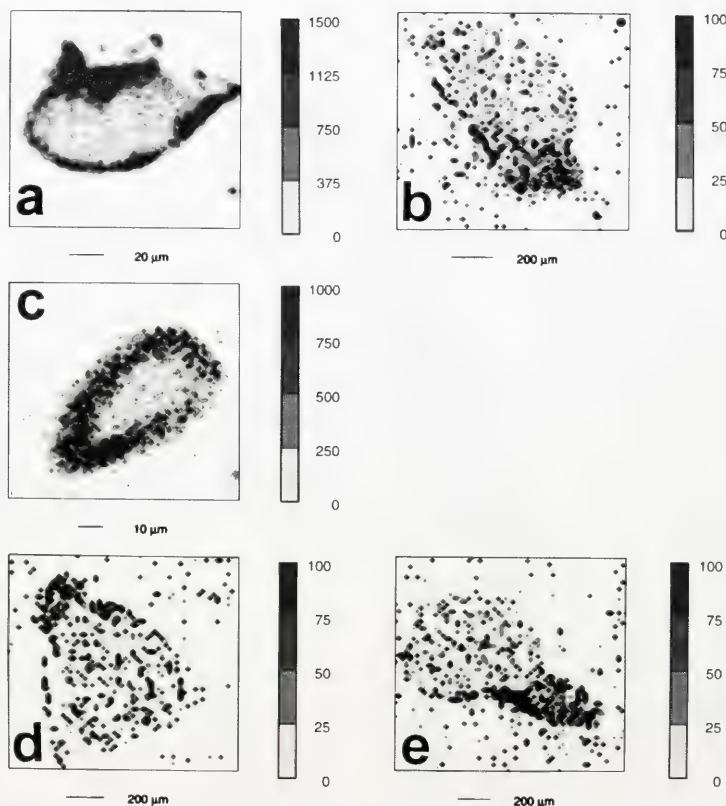


Figure 10. Zinc concentrations (values in ppm) in selected squid statoliths: (a) paralarva; (b) ML = 182 mm, ♀ V; (c) paralarva; (d) ML = 214 mm, ♂ III; (e) ML = 214 mm, ♂ II.

have a detectable influence on Ca and Sr levels in the statolith. If any effects did occur, they were on a scale below the resolution limits of the microprobe.

Other elements present

Many different elements have been investigated for various reasons in otoliths, mollusc shells, ostracods, and brachiopods by means of a proton microprobe (Gauldie *et al.*, 1992, 1995; Sie and Thresher, 1992; Coote and

Trompetter, 1995; Dai *et al.*, 1995; Nystrom *et al.*, 1995; Bruckschen *et al.*, 1995). The first PIXE results on squid statoliths were reported by Durholtz *et al.* (1995). The present study concentrated on Ca and Sr, but other elements such as Zn, Cu, and Br were also detected and, on the basis of their distribution within the statolith contour, were considered to be genuine parts of the statolith composition. Their concentration was highly variable (*e.g.*, Zn), and they were detected only in certain statoliths. Explanation and description of the presence-absence pat-

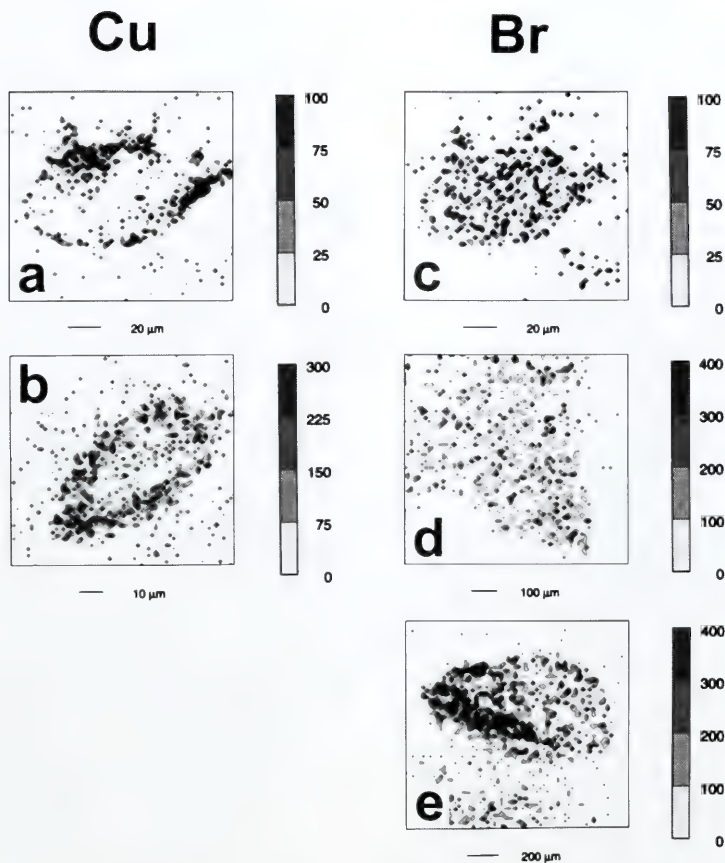


Figure 11. Copper and bromine concentrations (values in ppm) in selected squid statoliths: (a–c) paralarvae; (d) ML = 78 mm, ♂ I; (e) ML = 202 mm, ♂ II.

terms of these elements must await further investigation. It must also be pointed out that, because of the constraints of the method used, some elements of possible significance (*e.g.*, F; see Coote and Trompetter, 1995) were not investigated at all.

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Proteins of Morula-like Cells in Hemolymph of the Giant Clam, *Tridacna derasa*

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Abstract. The morula-like cell, a hemocyte packed with many large (about 3 μm in diameter) electron-dense granules, is found only in the hemolymph of giant clams belonging to the Tridacnidae. To clarify the function of the morula-like cell, we investigated its proteins, especially those found in the large granules. Proteins with molecular weights of 64 kDa, 17 kDa and 7.4 kDa were found to be specific to this type of hemocyte. N-terminal amino acid sequence analysis revealed that the 17-kDa and 7.4-kDa proteins were novel proteins rich in aromatic amino acids. Rabbit polyclonal antibody against a synthetic peptide of the 7.4-kDa protein reacted not only with that protein but also with a larger molecular weight (about 16-kDa) protein in the morula-like cell. Examination of the N-terminal amino acid sequences showed that the 16-kDa protein is distinct from the 17-kDa protein, and Western blot analysis suggested that it is a precursor of the 7.4-kDa protein. The zooxanthellate portion of clam mantle and kidney contained proteins immunoreactive to the antibody, but the azooxanthellate portion of the mantle did not contain any immunoreactive protein. These results suggest that the morula-like cells interact with the zooxanthellae.

Introduction

Giant clams, bivalve molluscs in the family Tridacnidae, harbor symbiotic zooxanthellae in the zooxanthellate tube, which arises from the stomach (Norton *et al.*, 1992). Carbon photosynthetically fixed by the algae is reportedly translocated to the animal (Goreau *et al.*, 1973). Why the zooxanthellae are not rejected by the host clam is an open question. To understand the mechanisms underlying the

stable association between the host and symbiont, it is important to study the self-nonspecific recognition system and the self-defense system of the host. In animals, blood cells, or hemocytes, participate in these systems.

Several types of hemocyte have been identified in the hemolymph of tridacnid species: two types in *Tridacna maxima* (Reade and Reade, 1976) and, more recently, three types in *T. crocea* (Nakayama *et al.*, 1997). The eosinophilic granular hemocyte in *T. crocea*, which corresponds to the type I cell in *T. maxima*, has phagocytic ability against foreign materials. The agranular cell in *T. crocea* seems to correspond to the bivalve hyaline cell reviewed by Cheng (1981, 1984). This type of hemocyte adheres to glass surfaces and contains electron-lucent granules and only a few electron-dense granules. The agranular cells were mainly found in the core space of the clots formed by the aggregation of hemocytes after exposure to seawater (Nakayama *et al.*, 1997). The morula-like cell in *T. crocea*, which corresponds to the type II cell in *T. maxima*, is packed with many large (about 3 μm in diameter), electron-dense granules. Hemocytes packed with large granules or globules are known as bivalve serous cells, or pigment cells (Cheng, 1981, 1984). The bivalve serous cells are yellowish or brownish and may participate in the exclusion of lipids. These cells seem to be different from the morula-like cell in *T. crocea* or the type II cell in *T. maxima*, both of which are colorless. The morula-like cell and the type II cell have been observed only in the Tridacnidae, which suggests that this type of hemocyte may participate in the maintenance of zooxanthellae in giant clams.

The giant clams obtain most of the organic carbon required for growth from the photosynthetic fixation of carbon by zooxanthellae (Klumpp and Griffiths, 1994). The clam has to collaterally supply minerals and bicarbonates to the zooxanthellae for photosynthesis; thus these materials must be transported to the algae (Trench,

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1987). Cytochemical staining has shown that the large granules in the morula-like cell do not participate in the transport of sugars and lipids (Nakayama *et al.*, 1997). To understand the role of the morula-like cell in giant clam-zooxanthellae symbiosis, the proteins specifically contained in the morula-like cell, especially those in the large granules, were studied.

Materials and Methods

Animals

Tridacna derasa were purchased from the Palau Mariculture Demonstration Center (Republic of Palau). In the laboratory on the research vessel *Sohgen-maru*, hemocytes were immediately collected as described below.

Preparation and fractionation of hemocytes

Hemolymph was withdrawn from the pericardial chamber with a 50-ml syringe and an 18-gauge needle, and collected in 50-ml polypropylene centrifuge tubes containing EDTA solution (0.04% EDTA-2Na and 2.5% NaCl, pH 7.4) as an anti-coagulation reagent. Hemocytes were collected by centrifugation ($700 \times g$, 5 min) and suspended in APSW, artificial Pacific seawater (Borowitzka and Larkum, 1976). The suspension of hemocytes was then layered on 50% Percoll (Pharmacia, Uppsala, Sweden) solution containing 2.5% NaCl. Morula-like cells were separated from eosinophilic granular hemocytes and agranular cells by centrifugation ($700 \times g$, 15 min). The morula-like cells were obtained as a pellet, and the mixture of the eosinophilic granular hemocytes and agranular cells remained in the upper layer. After suspension in APSW, the morula-like cells were partially disrupted by sonication (40 W, 3 min) on ice. The resultant solution was layered on 50% Percoll solution and centrifuged ($700 \times g$, 15 min). The pellet of Percoll density-gradient fractionation contained the debris of disrupted cells. The upper layer containing the large granules was collected, mixed with an equal volume of APSW, and recentrifuged to precipitate large granules.

Preparation of tissue extract

Tissues were dissected from the giant clam and extracted, using a Douce homogenizer, in Dulbecco's phosphate-buffered saline (Mg-, Ca-free). After removal of large debris by sedimentation, the extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of zooxanthellae

Zooxanthellae were isolated from the mantle of *T. derasa* by the method of Masuda *et al.* (1994). After disruption

of the mantle tissue by means of a Polytron-type homogenizer (Ystral GmbH, Dottingen, Switzerland), the extract was filtered through 10- μ m mesh. Zooxanthellae were contained in the filtrate and washed with APSW several times.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

After determination of protein concentration by a modified Lowry method (DC protein assay kit, Bio-Rad Labs., Hercules, California) according to the manufacturer's instructions, hemocyte and tissue samples were dissolved in SDS dissociation buffer (2.5% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) to approximately equal protein concentrations. After heating at 95°C for 10 min, samples were electrophoresed by PhastSystem (Pharmacia, Uppsala, Sweden), according to the method of Laemmli (1970), on 20% SDS-polyacrylamide gels.

Amino acid sequencing

Proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride (PVDF) membrane and then visualized with 0.5% Coomassie brilliant blue R-250 solution. The appropriate stained bands were cut out, and the N-terminal amino acids sequence was determined by a gas-phase amino acid sequencer (PSQ-2, Shimadzu Co., Kyoto, Japan).

Polyclonal antibody

A peptide with 15 amino acids was designed from the determined N-terminal amino acid sequence of the 7.4-kDa protein and synthesized by the multiple antigenic peptide (MAP) method (Posnett *et al.*, 1988). The polyclonal antibody was raised by immunizing rabbits with the MAP.

Western blot analysis

The hemocytes or granule lysate was applied to SDS-PAGE, then electrotransferred to PVDF membrane. Immunoblotting was performed according to the procedure of Towbin *et al.* (1979). After being treated with the blocking solution (Block Ace, Snow Brand Milk Products, Hokkaido, Japan), blots were incubated overnight at 4°C with the anti-7.4-kDa protein rabbit polyclonal antibody at 1:100 dilution. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel, Organon Teknica Corp., Durham, North Carolina) at 1:200 dilution or an ABC-AP rabbit kit (Vector Labs., Burlingame, California) was used as a secondary antibody. The immunoreactive band was visualized with a peroxidase staining re-

agents kit (Konica co., Tokyo, Japan) or an alkaline phosphatase substrate kit IV (Vector Labs., California).

After being electrotransferred to PVDF membrane, the glycoprotein was oxidized with periodic acid and reacted with biotin-hydrazide by the method of Kondo *et al.* (1991), and the sugars were visualized by avidin conjugated with horseradish peroxidase using a commercial kit (G. P. Sensor, Honen co., Tokyo, Japan) according to the manufacturer's instructions.

Analysis of amino acid sequence

An amino acid sequence homology search was performed using the MPsearch program underlying the Smith-Waterman sequence comparison algorithm (Smith and Waterman, 1981) through e-mail servers at the National Cancer Center (Tokyo, Japan).

Results

Proteins specific to the morula-like cell

Three types of hemocytes (the eosinophilic granular hemocyte, the agranular cell, and the morula-like cell) were recognized in the hemolymph of *Tridacna derasa* (data not shown), as in *T. crocea*. After fractionation of the morula-like cell in *T. derasa* and subfractionation of its granules, each lysate was analyzed by SDS-PAGE. Comparison of the SDS-PAGE patterns of the morula-like cell and a mixture of the eosinophilic granular hemocyte and the agranular cell showed that proteins with molecular weights of 64 kDa, 17 kDa, and 7.4 kDa were specific to the morula-like cell (Fig. 1, lane 3). The 17-

kDa protein was not found in the large-granule fraction of the morula-like cell but was in the cell-debris fraction (Fig. 1, lane 4). The 64-kDa and 7.4-kDa proteins were in both the cell-debris (Fig. 1, lane 4) and the large-granule fractions (Fig. 1, lane 5).

N-Terminal amino acid sequence

After electrotransfer of proteins from SDS-polyacrylamide gels to PVDF membrane, the 17-kDa and 7.4-kDa bands were cut out and placed in the sequencer. Twenty-five amino acids and 30 amino acids were identified in the N-termini of the 17-kDa and the 7.4-kDa proteins, respectively. Both proteins were rich in tyrosine and phenylalanine. In the 7.4-kDa protein, aromatic amino acids appeared at almost every 10 amino acids (Fig. 2).

Immunodetection of the 7.4-kDa protein

Western blot analysis revealed that the polyclonal antibody raised against a synthetic peptide corresponding to the 8th to 22nd amino acids of the 7.4-kDa protein was immunoreactive against that protein (Fig. 3). Another protein having a molecular weight of about 16 kDa was also immunoreactive with the polyclonal antibody (Fig. 3, lane 4). This 16-kDa protein was not detected in the large-granule fraction but only in the cell-debris fraction of the morula-like cell. Because the 16-kDa and 17-kDa proteins migrated closely together on SDS-PAGE, the 16-kDa protein appeared only as a contaminant in the 17-kDa protein. Careful examination of the HPLC chart from the sequencer revealed that the sequence of the 16-kDa protein was Y-D-X-S-F-X-K-X-R-X-X-X-F-X-A-D-P-A-S-X-Q-N-Y-Y-R. This was identical to the sequence of the 7.4-kDa protein excepting the unidentified residues, but distinct from that of the 17-kDa protein. The lysate of eosinophilic granular hemocytes and agranular cells did not contain immunoreactive proteins (Fig. 3, lane 2).

Distribution of the 7.4-kDa protein

To analyze the tissue distribution of the 7.4-kDa protein, tissue extracts were analyzed by the immunoblotting technique. The hemocytes (Fig. 4, lane 1), the zooxanthellate portion of mantle (Fig. 4, lane 2), and the kidney (Fig. 4, lane 7) contained the immunoreactive protein. A heavily reactive band was also observed near the dye front of electrophoresis of the zooxanthellate portion of the mantle. No immunoreactive protein was detected in the azooxanthellate portion of mantle, gill, foot, and adductor muscle.

Characterization of immunoreactive protein in zooxanthellae

After zooxanthellae were isolated from mantle tissue, they were analyzed by immunoblotting and glycosylation

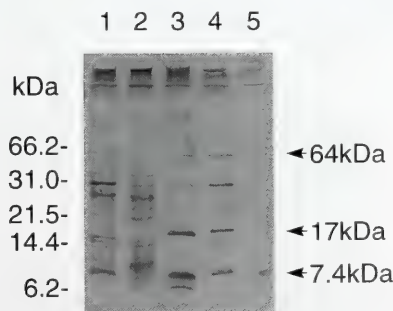


Figure 1. Identification of specific proteins in the morula-like cell. Hemocytes and fractionated samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and stained with silver nitrate. Lane 1, hemocytes lysate; Lane 2, lysate of the eosinophilic granular hemocyte and the agranular cell; Lane 3, morula-like cell lysate; Lane 4, precipitate of Percoll density gradient centrifugation after disruption of the morula-like cell; Lane 5, lysate of large granules which were isolated from the morula-like cell. Arrows indicate 65-kDa, 17-kDa, and 7.4-kDa proteins specific to the morula-like cell. Each lane contained 0.34 μ g protein except lane 5, which contained 0.1 μ g protein.

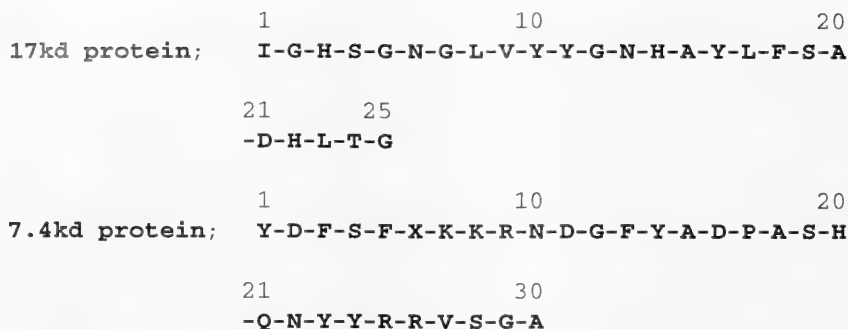


Figure 2. Amino acid sequences of the 17-kDa and 7.4-kDa proteins in the morula-like cell. The one-letter code is used. X, unidentified.

detection techniques. Immunoreactive protein appeared as a broad band and a slightly smaller component (ca. 5 kDa) that migrated faster than 7.4 kDa (Fig. 5A, lane 2). The 16-kDa and 7.4-kDa proteins in the morula-like cells (Fig. 5B, lane 1) and the immunoreactive protein in the zooxanthellae (Fig. 5B, lane 2) were shown to be glycosylated.

Discussion

Molluscan hemocytes packed with large colorless granules have been observed only in giant clams (the Tridacni-

dae). This type of hemocyte is known as a type II cell in *Tridacna maxima* (Reade and Reade, 1976) and a morula-like cell in *T. crocea* (Nakayama *et al.*, 1997). Proteins with molecular weights of 64 kDa, 17 kDa and 7.4 kDa were specific to the morula-like cell of *T. derasa* and not associated with the eosinophilic granular hemocyte or the agranular cell. The 7.4-kDa protein is contained in the large granules. No homologous sequence was found in the homology search of the N-terminal amino acid sequences of these proteins (Fig. 2), which indicates that these are novel proteins. The polyclonal antibody that was immunoreactive against the 7.4-kDa protein in the large granule was also immunoreactive with the 16-kDa protein in the cytoplasm of the morula-like cell. The N-terminal sequence of this 16-kDa protein was identical to that of

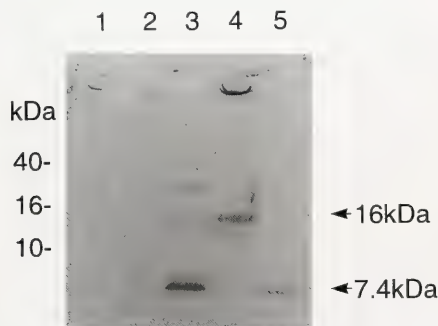


Figure 3. Immunoblot analysis of the 7.4-kDa protein specific to the morula-like cell. Hemocytes and the Percoll density-gradient fractions were electrophoresed, electrotransferred onto PVDF membrane, and immunologically stained. Lane 1, hemocytes lysate; Lane 2, lysate of eosinophilic granular hemocytes and agranular cells; Lane 3, morula-like cell lysate; Lane 4, precipitate of Percoll density gradient centrifugation of the morula-like cell lysate; Lane 5, the large granule lysate. Each lane contained 1.4 μ g protein except lane 5, which contained 0.4 μ g protein.

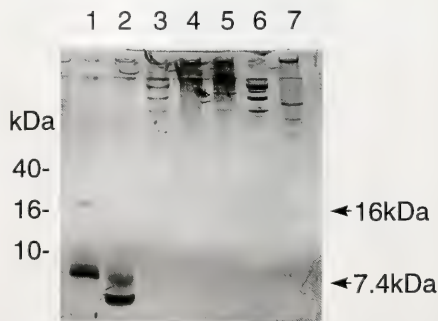


Figure 4. Immunoblot analysis of the 7.4-kDa protein in various tissues. Lane 1, morula-like cell; Lane 2, zooxanthellate portion of mantle; Lane 3, azooxanthellate portion of mantle; Lane 4, gill; Lane 5, foot; Lane 6, adductor muscle; Lane 7, kidney. Note that the zooxanthellate portion of mantle contained a large amount of immunoreactive proteins. Each lane contained 0.34 μ g protein.

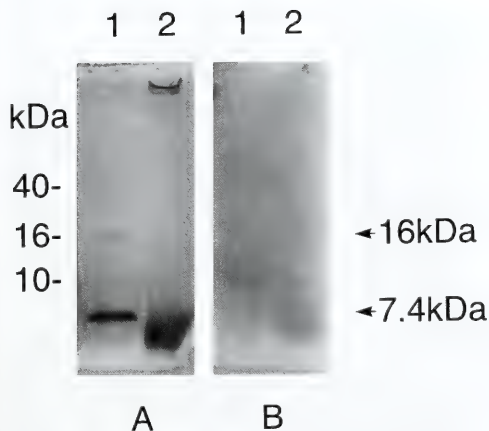


Figure 5. Immunoblot analysis and carbohydrate staining of the 7.4-kDa protein in the morula-like cell and zooxanthellae. (A) Immunological staining; (B) Detection of glycosylated proteins. Lane 1, morula-like cell; Lane 2, zooxanthellae. Each lane contained 1.4 μ g protein.

7.4-kDa protein but distinct from that of the 17-kDa protein. This suggests that this protein may be synthesized as 16 kDa. After transport into granules, the C-terminal region of the 16-kDa protein may undergo proteolytic maturation to 7.4-kDa. Detection of sugars by the biotin-hydrazide method indicates that 16-kDa and 7.4-kDa proteins are glycosylated.

The morula-like cells have been observed in several species of symbiotic clam in the families Tridacnidae and Cardiidae: *Tridacna maxima* (Reade and Reade, 1976); *T. crocea* (Nakayama *et al.*, 1997); *T. derasa*, *Hippopus hippopus*, *H. porcellunas*, *Corculum cardissa*, and *Fragum fragum* (our unpublished observations). The morula-like cell is always observed in the hemolymph of *T. crocea* and constitutes 6% of all the hemocytes (Nakayama *et al.*, 1997). This observation suggests that the morula-like cell may play a role in the giant clam-zooxanthellae symbiosis. Immunodetection showed that the amount of protein immunoreactive to the anti-7.4-kDa antibody and of a slightly lower molecular weight (ca. 5 kDa) was far greater in the surface layer of mantle containing zooxanthellae than in mantle tissue without zooxanthellae. The immunoreactive proteins were also detected in zooxanthellae and kidney, although their molecular weights were lower than 7.4 kDa. Some zooxanthellae grown in the zooxanthellal tube may be digested in the stomach or in the siphonal mantle (Maruyama and Heslinga, 1997). Other studies report finding zooxanthellae in the blood vessels of the kidney (Trench *et al.*, 1981) or in amoebocytes within the kidney (Morton, 1978). Although these

observations indicate that the 7.4-kDa protein may be in a digestive mass, the absence of phagocytic ability in the morula-like cell (Nakayama *et al.*, 1997) is evidence that the 7.4-kDa protein in these cells is not derived from phagocytic digestion of zooxanthellae. Nevertheless, the possibility that the morula-like cell takes up proteins secreted from eosinophilic granular hemocytes that digest zooxanthellae cannot be ruled out.

The 7.4-kDa protein was rich in aromatic amino acids in the N-terminal region and was characterized by alignment of aromatic amino acids at almost every 10th residue. Tyrosine-rich proteins have been reported in some invertebrates. The serous cell of *Crassostrea virginica* contains tyrosine-rich protein in the granules, which are composed of a complex of lipids and proteins with properties similar to those of lipofuscins (Cheng, 1981, 1984). The morula-like cell, although morphologically similar to the serous cell, lacks lipid in the large granules, as indicated by Sudan black B staining (Nakayama *et al.*, 1997). Thus the morula-like cell is not functionally similar to the serous cell. Tyrosine-rich proteins are also found in *Geukensia demissa* (Waite, 1977). The proteins, which are located in the shell cuticle, are suggested to be hardened by quinone tanning. When the hemolymph of *T. derasa* or *T. crocea* was mixed with seawater *in vitro*, the hemocytes aggregated to make large clots. In the aggregation process, some of the morula-like cells released the large granules, which burst and discharged their contents (Nakayama *et al.*, 1997). Although it is possible that the tyrosine residues in the 7.4-kDa protein are substrate for protein crosslinking, phenol oxidase activities have not been observed in the hemocytes and hemolymph of *T. derasa* or *T. crocea* (unpubl. data). The molecular weight of immunoreactive protein in the morula-like cell was larger than in the mantle. The 7.4-kDa protein may be synthesized in the morula-like cell, secreted in the mantle, bound to the zooxanthellae in the zooxanthellal tube, and finally, transported to the kidney with the zooxanthellae. Alternatively, the 7.4-kDa protein may be synthesized in the zooxanthellae and taken into the morula-like cell where it is modified by a process such as glycosylation, increasing its molecular weight. To clarify the function of the proteins specific to the morula-like cell, the complete amino acid sequences and the location of the genes coding these proteins must be determined.

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DNA-Dependent Protein Phosphorylation Activity in *Xenopus* Is Coupled to a Ku-Like Protein

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Abstract. DNA-dependent protein kinase (DNA-PK) is a nuclear enzyme and functions as a serine/threonine kinase that has been well characterized in both the human and the mouse. The regulatory subunit of DNA-PK is the Ku autoantigen. To demonstrate that a Ku-like protein is present in *Xenopus* oocytes, we used immunoprecipitation analysis with a monoclonal antibody raised against human Ku antigen and autoimmune serum containing anti-Ku antibodies. Metabolic labeling studies indicate that the Ku-like protein is synthesized mainly in late vitellogenic oocytes. By using a specific peptide substrate for DNA-PK, we demonstrate the activity of a DNA-dependent protein kinase in oocyte extracts. The kinase activity requires the Ku-like protein, since extracts depleted of Ku protein by immunoadsorption with human anti-Ku antibodies fail to demonstrate the DNA-dependent phosphorylation activity. The increased enzyme activity in vitellogenic oocytes may be correlated to the increased levels of Ku protein observed in these oocytes compared to the pre- and early vitellogenic oocytes.

Introduction

DNA-dependent protein kinase (DNA-PK) is a multi-subunit enzyme that includes the Ku protein, which is a heterodimer composed of 70-kDa and 80-kDa polypeptide subunits (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993) and a catalytic subunit of ~460 kDa (Blunt *et al.*, 1995). The Ku heterodimer functions as the regulatory component of DNA-PK and binds to the ends of non-

specific double-stranded DNA (dsDNA) (Gottlieb and Jackson, 1993). Although DNA-PK has been shown to affect multiple processes, including transcription (Feldmann and Winnacker, 1993; Cao *et al.*, 1994) and DNA repair and recombination (Anderson and Lees-Miller, 1992; Mizuta *et al.*, 1994; Taccioli *et al.*, 1994; Finnie *et al.*, 1995; and Peterson *et al.*, 1995), the *in vivo* targets of this enzyme have not been defined. Recent reports indicate that mice deficient in the 80-kDa subunit of Ku exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). These mice are also smaller than their normal littermates (Nussenzweig *et al.*, 1996).

DNA-PK activity has been detected in rabbit reticulocyte lysate; in eggs and oocyte extracts obtained from *Xenopus*, clam (*Spisula*), sea urchins (*Arbacia*); and in cellular extracts of mouse, hamster, and *Drosophila* (see review by Anderson and Lees-Miller, 1992 and reports by Walker *et al.*, 1985; Finnie *et al.*, 1995; and Kanungo *et al.*, 1996a). Much recent work has been done in *Xenopus*. Although the catalytic and regulatory subunits of DNA-PK remain to be characterized in this organism, DNA-dependent phosphorylation of histone during nucleosome assembly has been demonstrated in the oocytes (Kleinschmidt and Steinbeisser, 1991). DNA-PK has been reported to suppress RNA polymerase I transcription in extracts of embryonic kidney cells of *Xenopus* (Kuhn *et al.*, 1995; Labhart, 1995); and the N-terminal domain of *Xenopus* TATA box-binding protein has been shown to be a target of DNA-PK *in vivo* (Labhart, 1996). Furthermore, experiments with extracts of *Xenopus* eggs have indicated that DNA-PK may be involved in the phosphorylation of P1 protein (Someya *et al.*, 1995). We have carried out studies to determine whether the DNA-PK

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activity detected in *Xenopus* is associated with a Ku-like protein, and to evaluate preliminarily whether the enzyme activity varies in different stages of oocytes.

Materials and Methods

Unless indicated, all chemicals were purchased from Sigma. Female African clawed frogs (*Xenopus laevis*) were purchased from Nasco (Wisconsin), and the oocytes were staged according to Dumont (1972). Isolated oocytes were labeled with ^{35}S -methionine, 1 $\mu\text{Ci}/\mu\text{l}$ (Amersham) in Barth's modified saline (Gurdon, 1968). Defolliculated full-grown oocytes were homogenized in a Dounce homogenizer (50% v/v) in 50 mM HEPES, pH 7.4; 10 mM EGTA, 40 mM NaCl, 100 mM potassium acetate, 8.56 mM CaCl_2 , 2.29 mM MgCl_2 , 277 mM glycerol. Centrifugation of the homogenate ($12,000 \times g \times 30$ min, 4°C) yielded a supernatant that was recentrifuged to separate small particulate components from soluble components ($35,000 \times g \times 60$ min, Beckman SW 50.1).

Immunoprecipitation and protein analysis

Oocytes were homogenized in immunoprecipitation buffer (50 mM Tris-Cl, pH 7.5; 0.5 M NaCl, 0.05% NP 40) containing 1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{ml}$ leupeptin and 2.5 $\mu\text{g}/\text{ml}$ pepstatin. The homogenate was centrifuged ($12,000 \times g \times 30$ min) and the supernatant added to 2 mg of Protein A Sepharose CL-4B (Pharmacia) coupled to the appropriate antibody. The immunoprecipitates were boiled with protein sample buffer and resolved with SDS-PAGE, 7.5% (Laemmli, 1970) with subsequent autoradiography.

DNA-dependent protein kinase assay

A peptide comprising amino acids 11–24 of human p53 (EPPLSQEAFADLWKK) (Anderson, 1993) was used as the specific substrate for the DNA-dependent protein phosphorylation assay. The assay was performed at 20°C in a 15- μl reaction volume containing 10 μl of oocyte extract (140 μg total protein), 75 ng of the desired nucleic acids, 200 μM of peptide substrate, 2 mM MgCl_2 , 130 μM ATP, 1 mM dithiothreitol, and 10 μCi of γ - ^{32}P ATP (6000 Ci/mmol) (NEN, Du Pont). After 30 min, acetic acid was added to a concentration of 30%, and the reaction product was spotted onto P81 phosphocellulose discs (Whatman). The discs were washed in sequence with 30% acetic acid, 15% acetic acid, and acetone (5 min), and subsequently air dried and counted in a scintillation counter. Reactions without DNA and without substrate or with a nonspecific substrate were always included in the experiments. Subtraction of these values from reactions with added DNA and with added substrate gives the

final activity. A time-course experiment showed a linear incorporation of radiolabeled phosphate up to 30 min.

Immunodepletion of Ku from oocyte extracts

Oocyte extracts were depleted of Ku protein by immunoadsorption using Protein A Sepharose beads (Pharmacia) coupled with autoimmune sera (5 μl) containing anti-Ku antibodies and a monoclonal antibody raised against human Ku protein (mAb 162). Thirty microgram IgG (mAb 162) was used for each immunodepletion assay. The monoclonal antibody mAb 162 recognizes a conformational epitope on p80/p70 heterodimer. Therefore, it is effective for immunoprecipitation of native protein (the heterodimer) but is unable to recognize the denatured protein (Wang *et al.*, 1993). Mock-depleted extracts were prepared by treating the extracts with beads coupled to normal human serum. Depletion of other antigens, like Ro and Sm, was performed using the human autoimmune sera previously characterized.

Results

An autoimmune serum containing anti-Ku antibodies and monoclonal antibody 162 immunoprecipitated Ku-like polypeptides from radiolabeled oocytes (Fig. 1a, lane C, and Fig. 1b, lane B'). The polypeptides had electrophoretic mobilities closely approximating those of the Ku protein subunits identified in HeLa cells (Fig. 1a, lane A). Autoimmune sera containing antibodies to Ro and Sm but not to Ku were unable to immunoprecipitate a similar protein from the oocyte extracts (Fig. 1a, lanes D–E). Several other autoimmune sera containing anti-Ku antibodies were also examined. These sera immunoprecipitated Ku-like polypeptides (Fig. 1b) and in some cases additional proteins (Fig. 1b, lane D'). The monoclonal antibody 162 recognizes only the conformational epitope of the Ku heterodimer. Evidently, the conformational epitope for the Ku protein is conserved throughout evolution, because the *Xenopus* Ku protein was recognized by mAb 162, an antibody that detects the native form of Ku protein in mammalian cells. Unfortunately, mAb 162 does not recognize denatured Ku. Therefore an immunoblot analysis could not be performed with this antibody.

In *Xenopus*, the early vitellogenic phase represented by Y1–Y3 oocytes is followed by a vitellogenic phase represented by Y4–Y5 oocytes. When the oocytes are fully grown and no more yolk is deposited, the phase is designated as the post-vitellogenic stage (represented by Y6 oocytes). Immunoprecipitation assays on extracts prepared from radiolabeled oocytes at different stages of growth indicated that the biosynthesis of the Ku-like protein varied during oogenesis (Fig. 1c). The appearance of new protein was not detectable in early vitellogenic (Y1–Y3) oocytes (lanes A–C) but was detected in later stages

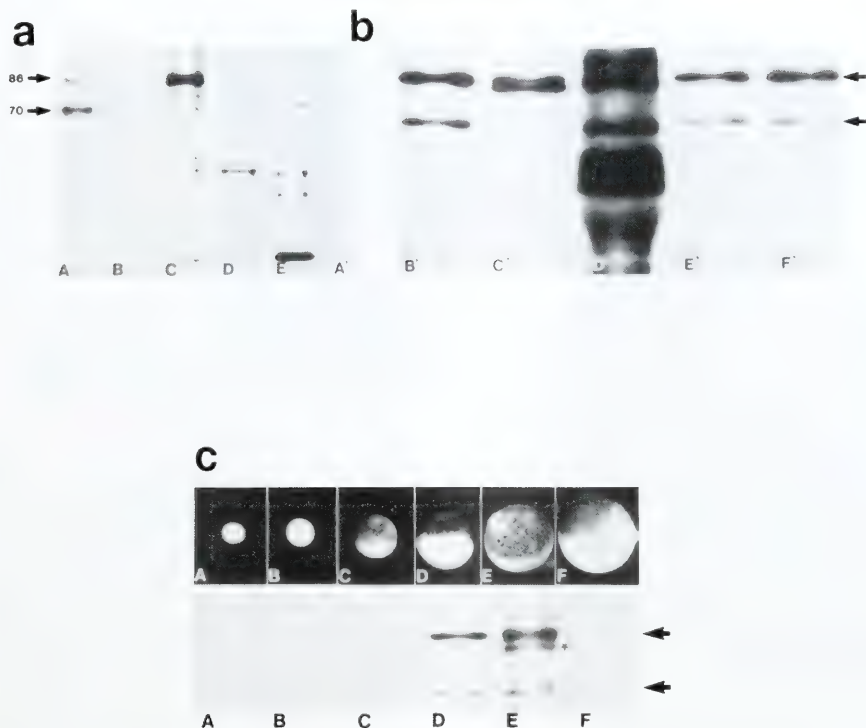


Figure 1. Autoradiogram of immunoprecipitates from oocyte extracts shows Ku-like polypeptides. Stage 5 oocytes were radiolabeled with [^{35} S]-methionine (see Materials and Methods) by incubating the oocytes for 18 h at 18°C. Ten oocytes were used for each immunoprecipitation assay. (a) Ku (p80/p70) immunoprecipitated with a monoclonal antibody (mAb 162) from HeLa cell extract (A); oocyte extracts immunoprecipitated with normal human serum (B), human autoimmune serum (KT) having anti-Ku antibody (C), human autoimmune serum having anti-Ro antibody (D), and human autoimmune serum having anti-Sm antibody (E). (b) Radioimmunoprecipitates of oocyte extracts with pre-immune IgG (A'), mAb 162 (B'), and with human autoimmune sera having anti-Ku antibodies; serum OM (C'), serum HT (D'), serum KT (E'), serum KY (F'). (c) Autoradiogram of immunoprecipitates from oocyte extracts shows Ku-like polypeptides during oogenesis. The OM serum, containing the anti-Ku antibody, was used for the immunoprecipitation assay. The number of oocytes for each stage was standardized so as to use equal amounts of total protein for each immunoprecipitation: Stage I (Y1) (Lane A), stage II (Y2) (Lane B), stage III (Y3) (Lane C), stage IV (Y4) (Lane D), stage V (Y5) (Lane E), and stage VI (Y6) (Lane F). The Ku polypeptides (p80 and p70) are indicated (arrows). Another protein band running slightly faster than p80 (*) is not identified, but may be a degradation product of p80. The unidentified band is not immunoprecipitated by mAb 162 or other autoimmune sera that recognize Ku (Fig. 1b).

(lanes D–E). We have not determined if the increased synthesis observed in the later stages of oogenesis correlates with a stage-specific function or if the oocytes stockpile DNA-PK for use after fertilization. The immunoprecipitated Ku is not as abundant in Y6 oocytes as in Y5 oocytes. This difference may be due to the decreased synthesis of Ku in the full-grown oocyte, Y6. The pre-

viously synthesized Ku apparently does not incorporate ^{35} S-methionine, and therefore the intensity of the Ku polypeptides in the radioimmunoprecipitation assay is less than that in Y4 and Y5 oocytes. Because the antibody does not recognize the Ku protein in immunoblots of crude extracts of oocytes, we could not directly quantify the level of Ku protein stored in the oocytes.

Using a specific peptide substrate (derived from the tumor suppressor protein p53), we detected a DNA-dependent phosphorylation activity in oocyte extracts prepared from oocytes at stages Y4–Y6. The peptide was phosphorylated in the presence of sonicated calf thymus DNA, but not in the presence of single-stranded (ss) DNA, total RNA from calf thymus or a 23-mer ss-oligonucleotide (Fig. 2a). This result suggests that double-stranded DNA (dsDNA) activates a kinase that is present in the oocytes. When oocyte extracts were pretreated by immunoprecipitation with anti-Ku antibodies, specific phosphorylation was reduced significantly. Patient sera containing anti-Ku antibodies removed most of the catalytic activity from the oocyte extract, whereas sera containing anti-Sm and anti-Ro antibodies had no significant effect (Fig. 2b). Thus, as with the activity observed in mammalian cells, the DNA-dependent protein kinase activity detected in the oocyte extracts appears to be regulated by a Ku-like protein.

In early vitellogenic oocytes (Y1–Y3), the Ku-like protein is barely detectable as determined by radioimmuno-precipitation. To assay stage-specific DNA-PK activity, we isolated the oocytes at different stages. Extracts prepared from early-stage oocytes had very little DNA-PK activity compared to the vitellogenic (Y4–Y5) and late-vitellogenic (Y6) oocytes (Fig. 3). This correlation between levels of Ku-like protein and DNA-PK activity suggests a functional link.

Discussion

These studies demonstrate, by using immunological and functional criteria, that *Xenopus* oocytes have a Ku-like protein that is associated with the activity of DNA-dependent protein kinase (DNA-PK). In sea urchins, we have observed a similar association of immunologically identical protein subunits (unpubl. data). Although Ku homologs from *Drosophila* (Jacoby and Wensink, 1996) and yeast (Feldmann and Winnacker, 1993) have been described, their association with DNA-PK activity has not been clearly documented. Nonetheless, it appears likely that this holoenzyme has been conserved through evolution. Cross-reactivity between a Ku-like protein in *Xenopus* oocyte extracts and the monoclonal antibody 162, which recognizes the conformational epitope (p80/p70) of the human Ku, supports this notion.

Our results demonstrate that the oocytes synthesize more Ku-like protein during the late vitellogenic phase. This suggests that Ku may be one of the maternal proteins that must be stockpiled in abundance for subsequent use in embryogenesis. In full-grown oocytes (Y6), therefore, the radiolabeling of the immunoprecipitated Ku protein is not as prominent as in Y4 and Y5 oocytes. This may

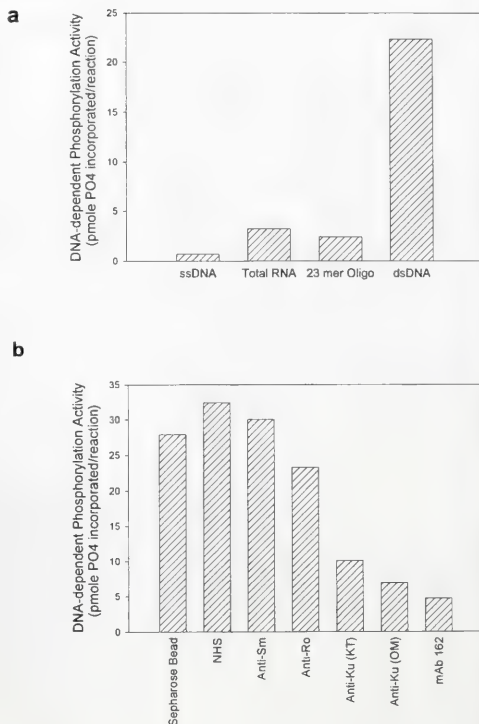


Figure 2. (a) Induction, using nucleic acids, of DNA-dependent protein kinase activity in oocyte extracts to evaluate the nucleotide-dependent enzyme activity. Values obtained from assays of extracts in the absence of any added nucleotide were subtracted from the values obtained from assays in the presence of different nucleotides. Similarly, specific peptide phosphorylation assays were performed by comparing assays with and without the addition of the peptide substrate. Enzyme activity in the crude extract is expressed as picomole phosphate incorporation per reaction containing 200 μ M specific peptide substrate; ssDNA (sonicated single-stranded calf thymus DNA), total RNA from calf thymus, 23 mer oligo (single-stranded oligonucleotide) and dsDNA (sonicated double-stranded calf thymus DNA); (b) Ku antigen-depleted oocyte extract shows a significant decrease in DNA-dependent protein kinase activity. The nucleic acid used to induce the kinase activity is sonicated calf thymus dsDNA. Human autoimmune sera with antibodies against Ku, a monoclonal antibody against human Ku (mAb 162), Sm and Ro were used to deplete the respective antigens from oocyte extracts; standard immunodepletion techniques were used. Normal human serum and Protein A Sepharose beads were used as controls.

be attributed to a slowing or cessation of synthesis of Ku in Y6 oocytes that have already stored the previously synthesized Ku. The low level of DNA-PK activity detected in the pre- and early vitellogenic stages of oocytes is consistent with the finding of minimal levels of the

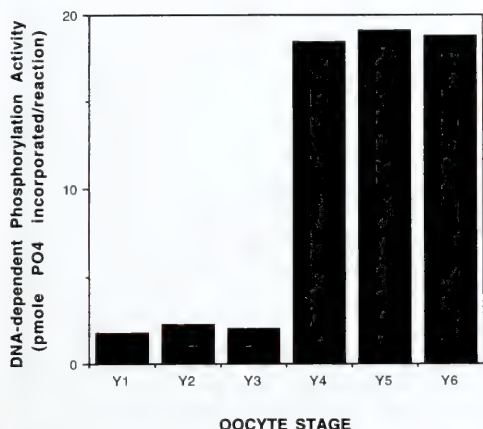


Figure 3. DNA-dependent protein kinase activity in oocytes at different stages. The isolated oocytes were homogenized as described in Materials and Methods (Immunoprecipitation and protein analysis). The total protein content of oocytes at each stage was equalized (140 μ g) in all the extracts used for DNA-PK activity assay. The specific peptide was used as the substrate.

Ku protein detectable in these stages. Induction, using dsDNA, of the kinase activity in the oocyte extracts distinguishes in *Xenopus* an enzyme (DNA-PK) that is functionally similar to the one reported in human cell lines (Carter *et al.*, 1990). The association of this activity with a Ku-like protein is confirmed with immunodepletion techniques when anti-Ku antibodies remove the activity from the oocyte extracts. Ku protein and DNA-PK catalytic subunit (p460) remain as a complex at physiologic salt concentrations, but dissociate at high salt (0.5 M) concentrations (Gottlieb and Jackson, 1993). Therefore, we used anti-Ku antibodies coupled to Protein A-Sepharose beads to immunodeplete the holoenzyme from oocyte extracts prepared in low salt buffers. Extracts incubated with these beads lost the DNA-PK activity, whereas other nonspecific antibodies did not affect the activity. These results indicate that the Ku protein is associated with the DNA-PK activity observed in the oocyte extracts.

Although considerable evidence indicates that DNA-PK plays a role in DNA repair and recombination (reviewed by Anderson, 1993), the *in vivo* targets of the DNA-PK remain largely unknown. Additional studies in clarifying the behavior of the enzyme during early embryogenesis may be useful in recognizing specific targets of this enzyme. We are investigating whether the post-fertilization nuclear translocation of the enzyme activity reported in the sea urchin (Kanungo *et al.*, 1996b) also takes place in *Xenopus*; the results should elucidate the enzyme's specific cellular functions during development.

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Ciliary Feeding Assisted by Suction From the Muscular Oral Hood of Phoronid Larvae

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Abstract. Phoronid larvae have an oral hood upstream from a postoral encircling array of ciliated tentacles. Cilia move water over the hood and between the tentacles, from anterior to posterior. When an algal cell or other particle in this current contacts a tentacle, the neighboring part of the hood lifts, and the particle is drawn toward the mouth. The correlation between movements of hood and particles indicates that the particle moves with water entering the enlarged space beneath the hood. Each lift of the hood is preceded by contact between a particle and a tentacle. A hood lift follows contact with a particle anywhere along the length of a tentacle, and clearance rates are thus proportional to the total length of tentacles deployed and the velocity of the current past the tentacles. After being detained at the ciliary bands of tentacles, particles are transported by the hood lift at speeds exceeding measured transport along the frontal ciliated surfaces of other larval forms. Faster transport may aid capture of faster prey. The larva's feeding mechanism is unique to the phylum Phoronida. Larvae of brachiopods, bryozoans, hemichordates, and echinoderms have similar ciliary bands producing feeding currents, but none are known to transport food toward the mouth by suction produced by muscle contractions.

Introduction

The actinotrocha is the feeding larval form of the lophophorate phylum Phoronida. There is conflicting evidence on homologies between structures of the actinotrocha and those of other larval forms. The actinotrocha resembles the deuterostome larvae of hemichordates and

echinoderms in the arrangement of the ciliary bands of its tentacles and in its monociliate cells (Garstang, 1962; Strathmann, 1973; Zimmer, 1973; Herrmann, 1976; Nielsen, 1987; Lacalli, 1990). Less obviously, it resembles spiralian trochophores and differs from deuterostome larvae in the direction of beat of preoral cilia on the margin of the oral hood (Lacalli, 1990). A close relationship between phoronids and spiralian has also been inferred from studies of 18s ribosomal DNA sequences (Halanych *et al.*, 1995). The inferred phoronid-spiralian relationship implies convergence between the larval and adult feeding apparatuses of the phoronids (and other lophophorate phyla) and those of larval enteropneusts and echinoderms (Halanych, 1996).

The phoronid actinotrocha differs strikingly from both spiralian and deuterostome larval forms in its large, mobile, and muscular oral hood, present throughout growth and development of the larva (Fig. 1). The anatomy and movements of this oral hood have been described, most recently in connection with descriptions of the nervous system (Hay-Schmidt, 1990a, 1990b; Lacalli, 1990). Though its structures have been studied extensively, the function of the oral hood remains unclear. Here we examine its role in the capture and transport of food.

Previous accounts of feeding by actinotrochas have focused on the operation of the lateral, laterofrontal, and frontal ciliary tracts on the tentacles. Paths of captured particles were compared with those observed for echinoderm larvae and prompted the suggestion that local reversals of groups of lateral cilia effected the capture of particles (Strathmann, 1973). It is also possible that the laterofrontal cilia of the tentacles play a sensory role in detection and capture of particles (Strathmann, 1973; Gil-mour, 1978; Hay-Schmidt, 1989; Lacalli, 1990). The increase in number and length of tentacles during larval

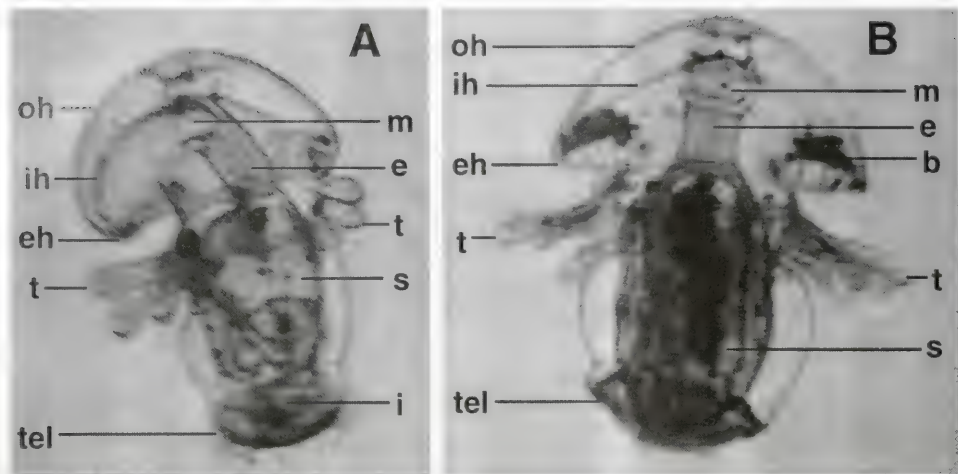


Figure 1. Actinotrochas of *Phoronopsis viridis* at the 12-tentacle stage in left and slightly dorsal view (A) and the 14-tentacle stage in ventral view (B). Labeled parts are mass of red blood cells (b), esophagus (e), edge of the oral hood (eh), intestine (i), inner wall of the oral hood (ih), mouth (m), outer wall of the oral hood (oh), stomach (s), tentacles (t), and telotroch (tel). The blood cells form paired masses in the hood and collar of the larvae at the 14-tentacle stage, and in Figures 2 to 5 should be distinguished from captured particles, such as the dark material in the stomach of the larva in (B).

development is like the increase in tentacles or lobes of other larval forms that use ciliary bands to capture food, and this increase has been interpreted as a means of increasing feeding capacity (Herrmann, 1979). These accounts all emphasize the role of cilia in capture of food.

Other observations suggest differences in the feeding mechanism. Actinotrochas can ingest large dinoflagellates and other prey that are large relative to the width of the tentacle (Lebour, 1922; Herrmann, 1976). Could the oral hood, in combination with propulsive and sensory cilia of the tentacles, provide a mechanism of particle capture that would account for these observations? The oral hood is lifted in response to a touch on the upper surface of the tentacles, and some ciliated cells of the tentacles are associated with nerve processes (Hay-Schmidt, 1989; Lacalli, 1990). These observations suggest that the tentacles might sense food and a lift of the oral hood aid in its capture. Here we describe transport of particles aided by movement of the oral hood, a process peculiar to the actinotrochas.

Materials and Methods

We examined actinotrochas of two types. Our first observations were of a single actinotrocha that was collected from the coastal plankton off Plymouth, England (English Channel), in June 1989. It matched descriptions of *Pho-*

ronis muelleri (Emig, 1982; R. L. Zimmer, pers. comm.), which occurs in this region. This larva was a more slender, graceful form than others that we observed.

Videorecordings were of actinotrochas from the northeastern Pacific Ocean. Several were collected from the plankton near Friday Harbor, Washington, USA (San Juan Channel), in the spring of 1991. These matched descriptions of the larvae of *Phoronopsis harmeri* (Zimmer, 1964). Zimmer described the anatomy of this larva in some detail under the provisional identification of Actinotrocha A. Others were collected from the plankton of Bodega Harbor, California, and may have belonged to *Phoronopsis viridis*, which is abundant there (Everett, 1991). These larvae resemble those of *P. harmeri*, and the species may be synonymous (Emig, 1982). Some of the actinotrochas collected near Friday Harbor were damaged in the posterior region, perhaps during collection, but swam and captured particles (Figs. 2 and 5). Those from Bodega Harbor were collected during a shorter plankton tow and were undamaged (Figs. 1, 3, 4).

Captured particles were the dinoflagellate alga *Prorocentrum micans* (about 20 μ m), plastic spheres (polystyrene divinylbenzene of 5 to 30 μ m diameter), and in a few cases Sephadex spheres from 25 to >65 μ m diameter. Particles were added by Pasteur pipette at a wide range of concentrations.

All measurements of rates were for larvae observed and videorecorded with a compound microscope at room temperature (about 15° to 20°C). For estimates of velocities, times were obtained either from a time-date generator that labeled recordings to the nearest 0.01 s or from the interval between frames; distances were obtained from recordings of a stage micrometer. (Two videorecorders were used, resulting in different frame intervals: 60 frames per second for *P. harmeri* at Friday Harbor, and 30 frames per second for *P. viridis* at Bodega Bay. Because the greatest velocities included estimates from 30 images per second, the greater velocities estimated from sequences of *P. viridis* did not result from differences in time intervals but rather from condition and behavior of larvae.) For most estimates of particle speeds, larvae were in chambers at least 1 cm in diameter by 0.5 cm deep, but because the larvae were often close to the upper or lower surface of the chamber, drag affected velocities, with greater effects expected at greater distance from a larva's motion. This chamber artifact is not so severe as to prevent comparisons of velocities close to moving cilia (Emlet and Strathmann, 1994). One estimate of the speed of particle transport was of a larva confined within the space in nylon plankton netting under a coverglass. Qualitative observations included larvae feeding in a water-cooled chamber under a dissecting microscope, with the larvae at least 1 cm from the nearest wall and temperatures close to ambient sea temperatures (about 10° to 15°C).

Results

Though different in form, actinotrochas from the plankton off Plymouth and those from Friday Harbor and Bodega Harbor used the oral hood in a similar way. Particles were carried past the actinotrocha in the current produced by the lateral cilia of the tentacles and a posterior ring of longer cilia (the telotroch). When a particle reached the area near the upstream side of the tentacles, the oral hood rapidly lifted, drawing the particle toward the mouth. When the hood lowered again, the particle remained beneath the oral hood (Figs. 2, 3, 4).

Hood lifts were more obvious in the larva from Plymouth (presumed to be *Phoronis muelleri*), but detailed quantitative observations were provided by videorecordings of captures by the larvae from Friday Harbor and Bodega Harbor (presumed to be *Phoronopsis harmeri* and *Phoronopsis viridis*). Hood lifts during some captures were inconspicuous but evident when videorecorded sequences were analyzed frame by frame. A hood lift followed a particle's contact with a tentacle—contact being defined as an approach within the length of the cilia. Close approach to the oral hood, as in Figure 2 at 0.1 s, did not trigger a hood lift. In all successful captures, the

particle first came close to the tentacle and then the hood lifted. Hood lifts were sometimes slight, but transport of a particle from a tentacle to the vicinity of the mouth was accompanied by a hood lift in every sequence in which the positions of both particle and hood were visible.

The hood was often raised from an initial slit-like opening of about 10 μm or less (Fig. 2, from 0 to 0.2 s), to 50 μm or more (Fig. 2, from 0.5 to 0.7 s). Thus space under the oral hood increased when the oral hood lifted, and an influx of water must have occurred.

Variation in particle speeds during a capture indicates that the particle is initially being detained at the tentacle before being transported proximally along the tentacle, at a higher speed, during the hood lift. In the videorecorded captures, a particle's speed increased as it approached the tentacle; after making contact, it usually moved slowly for a brief period, often moving to the side of the tentacle before being returned to the frontal surface of the tentacle. Movement along the tentacle toward the mouth increased in speed when the oral hood lifted. Once beneath the hood, particles were moved slowly toward the mouth, often with a pause on the way. The initial detention at the tentacle indicates that a ciliary mechanism for the initial capture and concentration of particles operates before the hood is lifted. The hood lift then assists the capture by aiding transport toward the mouth.

Estimated speeds of particles during several captures confirmed this pattern of movement. In a capture by *Phoronopsis harmeri* (Fig. 2, particle marked by a bar), the maximum speed as the particle approached the tentacle was 1.1 mm s^{-1} . After contact there was a prolonged period of little proximal movement, with maximum speeds less than 0.5 mm s^{-1} in the interval from 0.2 to 0.5 s. During this interval (at about 0.4 s) the hood began to lift. As the hood continued to lift, the particle's speed increased to 0.9 mm s^{-1} . In a second observation on the same larva (not shown), the particle's speed was less than 0.5 mm s^{-1} just after it had contacted the tentacle but increased to 1.1 and then 1.3 mm s^{-1} as the hood lifted.

In the capture by *Phoronopsis viridis* in Figure 3, the particle's maximum speed as it approached the tentacle was 1.6 mm s^{-1} . The particle moved little between contact with the tentacle, at about 0.07 s, and the beginning of transport proximally, at about 0.08 s. Then the particle traveled proximally at about 1.5 mm s^{-1} during the next 0.02 s, with the hood just beginning to lift. Its speed increased to 2.7 mm s^{-1} during the next 0.03 s of transport to the base of the tentacle (at 0.13 s in Fig. 3). In the capture in Fig. 4 (same larva), the particle moved lateral to the tentacle at about 0.1 mm s^{-1} (from 0 to 0.20 s) and then moved proximally at about 0.5 mm s^{-1} (from 0.20 to 0.27 s) before a noticeable hood lift. When the hood lifted, the particle moved proximally at about 2.4 mm s^{-1} (in the interval between 0.27 and 0.32 s). In a third capture

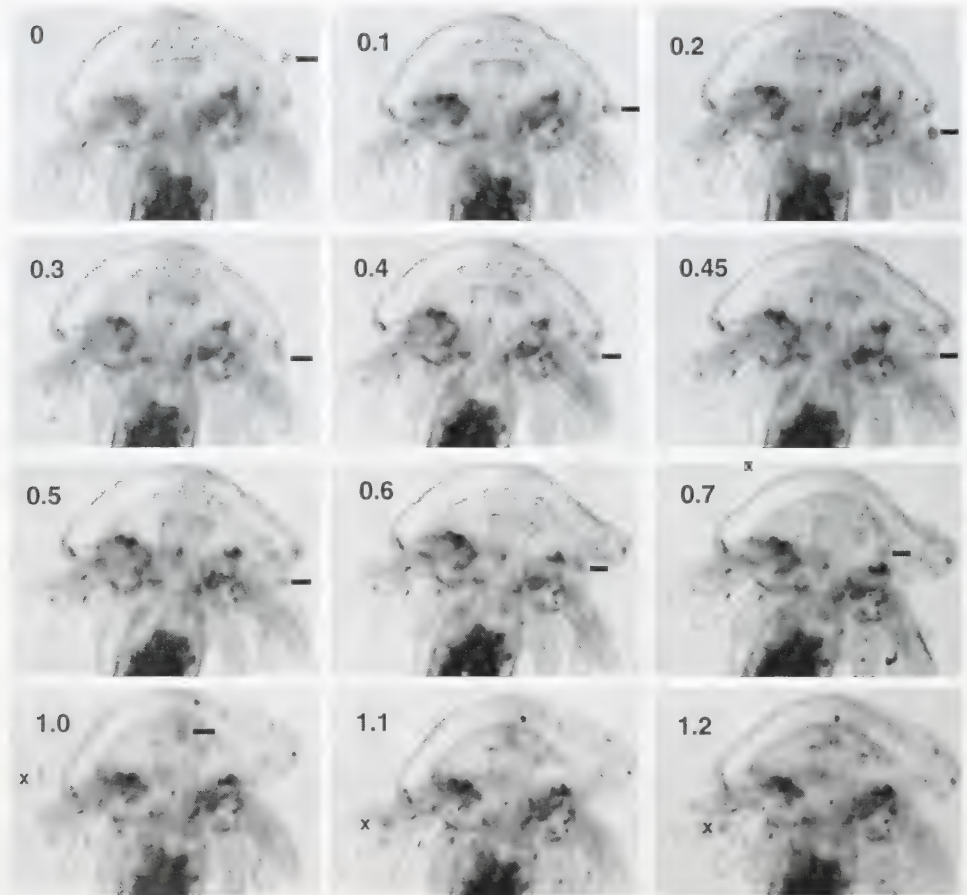


Figure 2. Two captures of the alga *Prorocentrum micans* by an actinotrocha (*Phoronopsis harmeri*) in ventral view. Numbers at the upper left of each frame are time in seconds from the first frame in the sequence. A black bar is to one side of the first algal cell captured on the viewer's right. An x is next to the second algal cell captured, on the viewer's left. The hood began to lift at about 0.4 s in the first capture, and the lift was nearly complete at 0.6 s. The cell had been drawn well under the hood at 0.7 s, and the second algal cell appeared (at the top of the frame). As the hood was lowered following the capture on the right, the second algal cell contacted a tentacle on the left at about 1.1 s, and a second hood lift and capture began on the left side. The width of the oral hood in the first frame is 480 μm . (This larva had sustained damage that extended the body posterior to the telotroch.)

(not shown and with hood movement out of focus and unrecorded), there was little movement during the first 0.2 s after the particle appeared near the tentacle tip, then movement lateral to the tentacle at less than 0.2 mm s^{-1} during the next 0.1 s, followed by proximal transport at 1.3 mm s^{-1} during the next 0.05 s. With another larva of

P. viridis (confined in a nitex mesh cage), a plastic sphere was held on the tentacle with little or no motion, then transported proximally along the tentacle at 0.25 mm s^{-1} during 0.1 s; it halted again for 0.1 s, and then increased speed to 0.6 mm s^{-1} as the hood lifted.

Movements of particles corresponded even to irregular

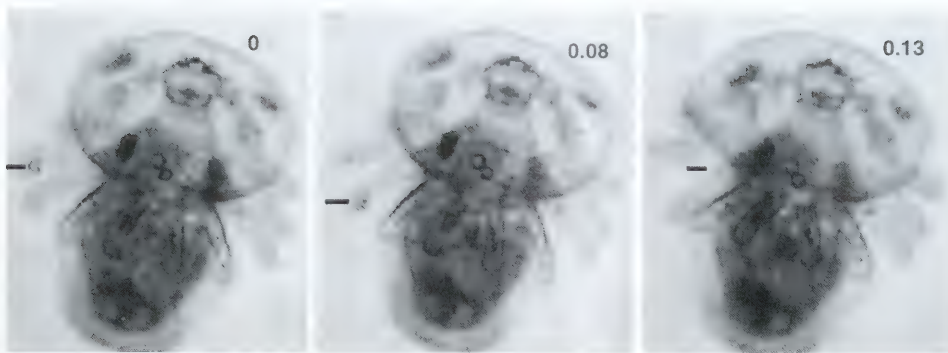


Figure 3. Capture of a plastic sphere by an actinotrocha (*Phoronopsis viridis*) in ventral-anterior view. The black bar is to one side of the plastic sphere. Numbers at the upper right of each frame are time in seconds from the first frame in the sequence. The sphere contacted the tentacle near the tip and moved most of the length of the tentacle during the hood lift between 0.08 and 0.13 s. The width of the oral hood in the first frame is 460 μm .

lifting and lowering of the hood. When an actinotrocha lifted and lowered its oral hood in rapid succession, a particle moved proximally with each lift and distally with each lowering (Fig. 5). The motion of the particle in this and other examples exactly followed the motion of the hood.

The oral hood sometimes lifted when no particle was captured. When larvae were disturbed, they often raised the hood far beyond the elevation that accompanied particle captures. Lifting the oral hood may serve other functions, such as rejection of particles, escape, or defense, but our observations of the close correspondence between encounters with particles and elevation of the oral hood indicated a response to individual particles and a role in captures. The improbability of hood lifts and captures coinciding by chance alone was demonstrated by analysis of videorecorded sequences of 10-s duration with an actinotroch of *P. viridis* capturing plastic spheres 20 μm in diameter. In the first of these sequences, four plastic spheres passed close to the tentacles. Two passed with a hood lift and were captured. One passed with a hood lift but no capture. One passed without a hood lift. The observed proportion of particles passing that coincided with a hood lift was 0.75, and the proportion of particles captured was 0.5. The proportion of particles passing that would coincide with hood lifts by chance alone can be estimated by assuming that a capture or a hood lift each occurs within 0.3 s. The fraction of the interval with particles passing close to tentacles was then $4 \times (0.3 \text{ s}) / (10 \text{ s}) = 0.12$, and the fraction of the interval with hood lifts was $3 \times (0.3 \text{ s}) / (10 \text{ s}) = 0.09$. The probability of passage of a particle coinciding with a hood lift is then 0.011.

In a second sequence, also of 10 s but with a higher concentration of spheres, four spheres passed close to tentacles. Two were captured with hood lifts. One was not caught, but there was a hood lift. The fourth passed without a hood lift. There were also two motions of the hood with no particle in view. The observed fraction of particles passing that coincided with a hood lift was 0.75 and the observed fraction with captures was 0.5. Again, the expectation of this happening by chance alone can be estimated from the fraction of the interval with particles passing (about 0.12) and the observed fraction of the interval with hood lifts (about 0.15). The probability of a particle passing and a hood lift occurring together by chance is then 0.018. Even here the probability of the four passing spheres coinciding with hood lifts by chance is much lower than the observed proportion of coincident events. These calculations overestimate the probability of chance coincidence of hood lifts and captures because movements of hood and particle were more closely correlated than in the loose assumptions of our calculations, both in timing and in correspondence of the portion of the hood lifted to the position of the particle. The association of hood lifts and captures was not a chance occurrence.

When actinotrochas had ingested many particles, more particles passed the tentacles without a hood lift, and when they were disturbed, there were more hood lifts without the presence of a particle.

Lengths of cilia of actinotrochas of *Phoronopsis viridis* at the 14-tentacle stage were measured from video images. The four estimates of length of cilia on tentacles were 23, 25, 26, and 29 μm , within the range of cilium

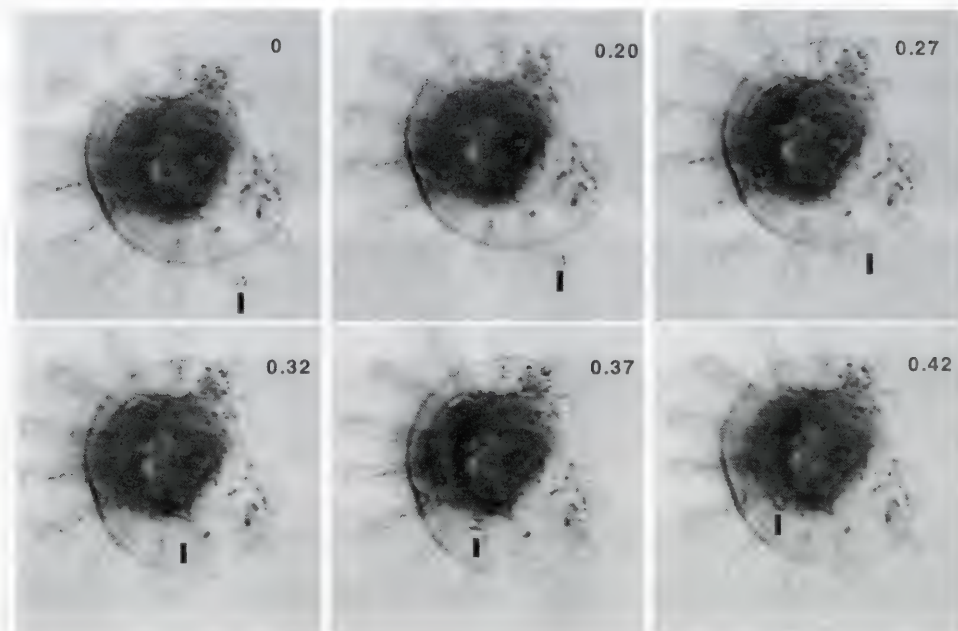


Figure 4. Capture of a plastic sphere by an actinotrocha (*Phoronopsis viridis*) in anterior view. The black bar is below the plastic sphere. Numbers at the upper right of each frame are time in seconds from the first frame in the sequence. The sphere approached and contacted the tentacle between 0 and 0.27 s. The hood began to lift at about 0.27 s, with the change in shape of hood evident at 0.32 s. Between 0.27 and 0.32 s the particle was drawn rapidly under the hood. It then moved more slowly toward the mouth while the hood remained lifted (at 0.37 and 0.42 s). The width of the oral hood in the first frame is 465 μm .

lengths observed for the adults of *Phoronis vancouverensis* (Strathmann, 1973). Estimates of cilium lengths from an arrested telotroch ranged from 51 to 57 μm (median 53 μm , $n = 7$). Estimates of cilium lengths from a beating telotroch ranged from 50 to 61 μm long (median 56 μm , $n = 7$). Tentacles (not including the cilium length) were 30 to 33 μm wide.

The largest particle found in the stomach of an actinotrocha of *P. viridis* was a Sephadex sphere 55 μm in diameter, but we did not test for the maximum size that could be captured and ingested.

Discussion

Roles of cilia and hood lift in capturing particles

The demonstrated role of the oral hood in transport of particles does not preclude the presence of ciliary mechanisms for particle capture similar to those in other larval forms. Our study focused on the role of the oral hood, but movements of particles suggest that cilia play an es-

sential role in capture and transport of particles. Cilia bring particles to the tentacles, initially detain them there, and aid in transporting them. The hood lift supplements a ciliary feeding mechanism but has not replaced it.

The actinotrochas appear to have two active responses to particles, with the ciliary one acting before the hood lifts. Cinefilms of particle capture by adult bryozoans have demonstrated that changes in the beat of the lateral cilia on the tentacles are associated with retention and transport of particles (Strathmann, 1982). One hypothesis of how the cilia of the tentacles of actinotrochas detain particles before the hood lifts is that particles approaching the tentacles induce the lateral cilia of the tentacles to arrest or reverse beat. This is consistent with the observed movements of particles to a position lateral to the tentacles on initial contact. There is a pause or slowing of movement of the particle at that location and then a transfer toward the frontal surface. Our videorecorded images of actinotrochas showed the paths of particles but were insufficiently clear to directly demonstrate changes in the beat of the cilia.

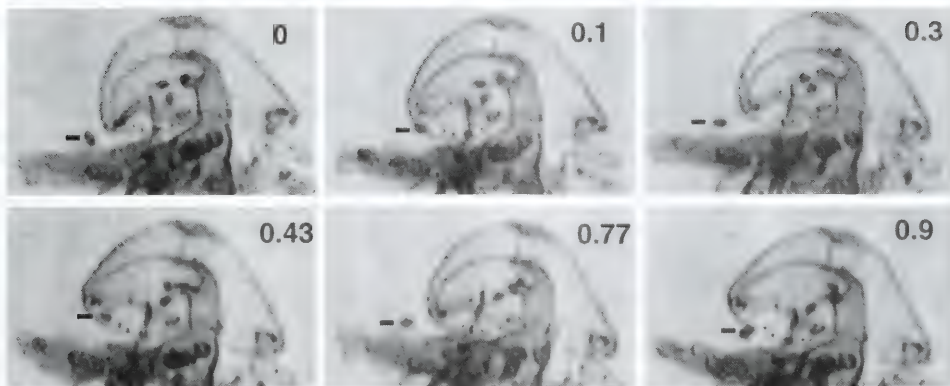


Figure 5. Movements of a cell of *Procoentrum micans* in relation to repeated hood lifts by an actinotrocha. The view is of the dorsolateral side of the larva. The black bar is to one side of the algal cell. Numbers at the upper right of each frame are time in seconds from the first frame in the sequence. The hood had completed lifts at 0.1, 0.43, and 0.9 s. In each case the algal cell was drawn inward to the edge of the hood. Lowering of the hood had produced maximum outward excursions of the algal cell at 0.3 and 0.77 s. The scale was undetermined. (This larva had sustained damage that shortened the body posterior to the tentacles.)

A second hypothesis is that particles are halted by a sieve formed by stationary laterofrontal cilia, as observed for the cyphonautes larva of bryozoans (Strathmann and McEdward, 1986). The actinotrochas do have laterofrontal cilia (Hay-Schmidt, 1989), but our videorecorded images were insufficiently clear for direct observation of sieving by those cilia. The laterofrontal cilia of the tentacles may be sensory (Strathmann, 1973; Gilmour, 1978; Hay-Schmidt, 1989; Lacalli, 1990; Pardos *et al.*, 1991). Instead of or in addition to acting as a sieve, the laterofrontal cilia could detect the particles and trigger the hood lift.

Our observations do not separate the effect of a hood lift from the effect of the cilia in transporting particles toward the mouth. Raising the oral hood could change the currents generated by the cilia on the hood margin and other cilia of the oral region by changing distances from the body wall and other cilia. It is also possible that changes in ciliary beat coincide with movements of the oral hood. Thus changes in ciliary currents could contribute to a change in the movement of a particle when the hood lifts. However, the simplest explanation is that the hood itself is moving the water and the particle with it during its lift because (1) changes in the volume of the space under the hood must create a flow of water and (2) movements of particles corresponded exactly with lifts of the hood.

Particles are retained in some manner when the hood is lowered. The cilia beneath the hood may aid retention as the hood is lowered. Possibilities include sieving as a

result of action and positions of cilia, changes in ciliary beat in response to particles, and changes in beat coordinated with movement of the hood.

Implications for quantitative performance

The hood lift of the actinotrochas is distinctive. Does it confer distinct advantages in either range of available foods or maximum clearance rates?

The hood lift may permit capture of more active prey than is possible when captures are by cilia alone. The greatest velocities of particles transported by hood lifts were 2.7 and 2.4 mm s⁻¹ in our study. Other measured velocities were slower and overlapped estimates for transport by cilia alone on animals with ciliary bands like those of actinotrochas. These estimates are 1.2 mm s⁻¹ for a particle transported down the arm of a pluteus (Hart and Strathmann, 1994), about 0.7 mm s⁻¹ in the food groove of the hemichordate *Planctosphaera pelagica* (Hart *et al.*, 1994), and 0.75 to 1.55 mm s⁻¹ for transport down a bryozoan's tentacle (Strathmann, 1982). In the mitraria of oweniid polychaetes (a larva with opposed bands but simple cilia), transport in the food groove was 0.1 to 0.4 mm s⁻¹ (Emlet and Strathmann, 1994). Transport by either hood lift or ciliary tracts should be adequate to overcome the 0.1 to 0.5 mm s⁻¹ swimming speeds of dinoflagellates (Sournia, 1982). Other potential prey swim faster. Reported swimming speeds for a variety of ciliates range from 0.5 to 1.2 mm s⁻¹ (Sleigh and Blake, 1977).

These comparisons suggest that the faster transport with some hood lifts could extend the range of prey that (after the initial encounter) can be retained and transported to the mouth.

The actinotrochas may capture prey larger than those available to other larvae with similar ciliary bands. Reported gut contents include armored dinoflagellates, tintinnids, diatoms, occasional bivalve and gastropod larvae, and a copepod egg (Lebour, 1922). Actinotrochas of *Phoronis muelleri* filled their guts with the armored dinoflagellate *Peridinium trochoideum* and even attempted to ingest smaller actinotrochas (Herrmann, 1976). In our study, the largest particle ingested by *Phoronopsis viridis* was only 55 μm , but this is 10 μm larger than the largest spheres ingested by bryozoan and echinoderm larvae under similar conditions (McEdward and Strathmann, 1987). The muscular oral hood of the actinotrocha provides a mechanism for capturing large prey, but such prey can also be captured by some ciliary mechanisms. Some types of small ciliated larvae ingest large particles. These include several types of polychaete larvae that do not use the opposed-band feeding mechanism (Strathmann, 1987). For example, a trochophore of a polynoid polychaete, 18 days old and thus only about 130 μm long, was able to capture a centric diatom 60 μm in diameter (Phillips and Pernet, 1996). The hood lift may provide capture of types of prey that would otherwise be unavailable, but this hypothesis was suggested, not tested, by our study.

Does capture by a hood lift permit a greater maximum clearance rate? The maximum clearance rate of actinotrochas depends on diversion of a particle and a small quantity of surrounding water from the much larger volume of water that flows within range of its sensors. When there is no particle to be caught, water flows posteriorly in the currents produced by the lateral cilia of the tentacles and by the posterior ring of cilia, but when a particle is detected by cilia or other structures on the tentacles, the oral hood lifts and the particle is captured together with a small quantity of water. Water may be eliminated as the oral hood is lowered, but the greatest concentration of suspended food comes not from filtration but from sensing and capturing individual particles. Such mechanisms have been termed scan and trap (LaBarbera, 1984) or active response (Strathmann, 1987) methods of suspension feeding. Particles contacting the tentacles anywhere from near the base (Fig. 2) to near the tip (Fig. 3) can be captured. Thus the maximum clearance rate depends on three distinct features: the speed of the water flowing past the tentacles, the length of the tentacles, and the distance from the tentacles at which particles are sensed. Is there evidence that the actinotrocha has evolved an unusual capability in any of these three components of clearance rate, as a possible result of transport aided by a hood lift?

If the hood lift permits captures from a faster current, then it could confer a greater clearance rate than is possible with ciliary bands alone. The actinotrocha might achieve a faster swimming and feeding current by additional propulsion from its telotroch, a posterior ring of longer cilia (Nielsen, 1987). Also, the lateral cilia themselves might produce a faster current. Our observations were not designed to measure velocities of particles approaching the tentacles, but the observed speeds of 1.1 and 1.6 mm s^{-1} were similar to the mean speeds of 0.8 to 1.4 mm s^{-1} recorded for a variety of echinoderm larvae (Hart, 1996). If actinotrochas produce feeding currents that are faster than those of similar larval forms, we failed to observe them. Also, in at least some captures the particle is detained on the tentacle for a brief period before the hood lifts. In these cases processes other than the hood lift are responsible for initially detaining particles (as discussed above).

Has capture aided by the oral hood resulted in evolution of a greater total length of tentacles and ciliary bands than in similar larval forms? The larva in Fig. 4 had a total length of tentacles of about 2.1 mm, if one assumes that all tentacles are parallel to the plane of focus; and because there are ciliary bands on both sides of each tentacle, the total length of band is about 4.2 mm. Echinoderm larvae also develop through a stage with this band length (Hart and Strathmann, 1994; Hart, 1996). Although differences in body organization prevent exact comparisons of body size, some echinoplutei with 6 to 8 arms are similar to this actinotrocha in both body length and ciliary band length (McEdward, 1984, 1986). The total length of lateral ciliary band of this actinotrocha is not extraordinary for larvae of its size.

It is possible that contact between a particle and the laterofrontal cilia triggers a hood lift, and the laterofrontal cilia may extend farther from the tentacle than the lateral cilia (Strathmann, 1973), thereby increasing the clearance rate. This hypothesis is subject to the same objection that particles can be detained at the tentacles before the hood lifts. If particles passing at a greater distance are retained, other processes than the hood lift may be responsible for this capability.

The hood lift does not appear to permit greater clearance rates, although there remains the possibility that the hood lift permits capture of prey that are so swift that it is the prey's speed rather than the actinotroch's speed that determines frequency of encounters.

Contrast with other larval forms

The use of muscles in connection with capture or transport of particles to the mouth is not known for other larvae that capture particles upstream from bands of simple cilia. A functional and structural equivalent of the oral hood is

unknown for the larval forms most closely resembling the actinotrocha. In the larvae of bryozoans, enteropneusts, and echinoderms, muscular movements pass food through the gut after it has been concentrated in the oral cavity by cilia (Strathmann, 1971, 1973; Strathmann and Bonar, 1976). Muscles of larval echinoids and ophiuroids dilate the mouth as an aid to rejecting particles, and dorsal muscles in asteroid larvae flex the larval body, which both opens the oral region as an aid to rejecting particles and changes direction of swimming (Strathmann, 1971). A functional analogue of transport aided by a hood lift is unknown for larvae of other lophophorates, of echinoderms, or of enteropneusts.

Transport aided by the oral hood also differs from particle capture by larvae with the opposed-band mechanism, which is widespread among larvae of the spiralian phyla. Veligers and trochophores with opposed bands rely on the action of cilia rather than on muscles for capture, transport, and rejection of particles (Werner, 1955; Strathmann *et al.*, 1972; Gallagher, 1988; Hansen, 1991). Polychaete larvae and their feeding mechanisms are diverse, however, and muscles aid capture or transport of food in some families. Reported examples include flexing of the chaetopterid larval body to ingest a mucous net (Werner, 1953), and capture of animal prey by tentacles of magelonicid larvae (Wilson, 1982), but these structures and activities do not resemble the action of the actinotrocha's oral hood.

The action of the actinotrocha's oral hood in feeding explains a prominent and peculiar feature of a distinctive larval form. The large number of muscles in the oral hood is also distinctive and may be required for the local lifts in response to particles contacting different tentacles. There is no known analogue in larvae of other phyla.

Adult forms also provide no close analogue to the hood lift of actinotrochas. Tentacle flicks by adult bryozoans aid transport of particles toward the mouth (Strathmann, 1982), but this does not appear to be a suction mechanism. The structure is not homologous to the actinotrocha's hood, and the mechanism is not analogous to the hood lift. (The tentacles of actinotrochas often move during particle capture, but it is not apparent that these twitches aid transport of particles.) Production of a feeding current by flapping one valve has been suggested for extinct strophomenide brachiopods, but the proposed function of flapping was production of feeding currents, not transport of particles from tentacles to mouth (Rudwick, 1970).

Suction by the oral hood of actinotrochas invites comparison with suction and ram feeding by fishes, but the particle velocities associated with hood lifts in our observations are far lower. A particle's velocity relative to the mouth of a small fish can be the combined effects of ram (movement of the fish toward the particle) and suction (Coughlin, 1994). For carp of 5 to 8 mm body length,

movement of prey relative to the mouth can be 260 mm s^{-1} (Drost, 1987), about 100 times the speed at which prey are moved by the actinotrocha's hood lift. The Reynolds number for flow produced by an actinotrocha lifting its hood is less than one. Thus, in contrast to ram and suction by small fish, viscous forces exceed inertial forces.

Acknowledgments

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Sexual Modes in the Colonial Kamptozoan Genus *Barentsia*

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Abstract. Sexual mode in colonial animals is expressed at the zooid, colony, and genet level; all three must be characterized to understand sexuality in these animals. I carried out such an examination of the sexual mode of a colonial kamptozoan (entoproct), *Barentsia hildegardae*, at Friday Harbor, Washington. Calyces never contained both ovaries and testes, and colonies never contained both male and female calyces. Calyces and colonies (including replicate colonies from the same genet) monitored over two years did not change sex. These results suggest that *B. hildegardae* is comprehensively gonochoric. For comparison, I examined the sexual mode of five other species in the genus *Barentsia*. *Barentsia benedeni*, *B. conferta*, and *B. ramosa* also appear to be comprehensively gonochoric. *Barentsia discreta* is hermaphroditic at the colony level, with gonochoric calyces whose sex is environmentally determined, as noted by previous workers. *Barentsia aggregata* has simultaneously hermaphroditic calyces; this was reported by the authors who described it, but has escaped notice in subsequent reviews of kamptozoan biology. There are thus three contrasting modes of sex within the genus *Barentsia*. All three modes also occur in colonial cnidarians, and two of them are known in bryozoans, colonial hemichordates, and colonial urochordates. These disparate sexual modes may have evolved as adaptations to differing environmental conditions or population densities.

Introduction

Colonial animals display a diversity of sexual modes. Every colonial species can be examined at two levels, that of the zooid (the structure that is iterated to form a colony) and that of the colony. In many colonial species,

there can be multiple colonies per genet (genetic individual: all tissue derived mitotically from one zygote) produced early in development by polyembryony, or later by fragmentation or encapsulation. Such animals have three levels of organization: the zooid, the colony, and the whole multicolony genet. One way of classifying the sexual modes of colonial animals (Wasson and Newberry, 1997) is to designate each of the three organizational levels as gonochoric (G), sequentially hermaphroditic (S), or simultaneously hermaphroditic (H). According to this system, 10 modes of colonial sex are possible (Fig. 1), although only 5 have known examples among animals (Wasson and Newberry, 1997).

Few researchers investigating colonial animals have thoroughly examined the sexual mode of zooids, colonies, and whole genets of a single species. Many report only the sexual mode of the zooids, leaving uncertain the sexual mode of the colony or the whole genet. We cannot examine the ecology and evolution of mating systems in colonial animals if words such as gonochoric and hermaphroditic are used without specifying the level to which they apply. Furthermore, colonial animals have rarely been monitored over time, so it has been difficult to distinguish gonochorism from sequential hermaphroditism (hermaphroditism with discrete, separated periods of male and female maturity) at any level.

The purpose of this investigation was to describe thoroughly the sexual mode of one barentsiid kamptozoan (entoproct), *Barentsia hildegardae*, by examining all three levels of colonial organization and monitoring them over time. Barentsiids are small marine suspension feeders; barentsiid colonies superficially resemble those of hydroids. Cuplike calyces containing U-shaped guts and ringed by tentacles are borne on stalks arising from prostrate stolons. The calyx is the sexually reproductive unit of a barentsiid kamptozoan zooid, at sexual maturity containing testes, ovaries, or both. Although the kamptozoan

GENET	Gonochoric	Sequentially hermaphroditic			simultaneously Hermaphroditic					
COLONY	gonochoric	gonochoric	seq. hermaphroditic		gonochoric	seq. hermaphroditic		simultaneously hermaphroditic		
ZOOID	Gonochoic	Gonochoic	Gonochoic	Seq. herm.	Gonochoic		Seq. herm.	Gonochoic	Seq. herm.	sim. Herm.
mode	GgG	SgG	SsG	SsS	HgG	HsG	HsS	HhG	HhS	HhH

Figure 1. Ten potential sexual modes of colonial animals. The three-letter abbreviation for each represents the mode of the genet (first capital letter), the colony (lower case), and the zooid (second capital), respectively. Only modes GgG, SsS, HhG, HhS, and HhH have known examples among colonial animals (Wasson and Newberry, 1997).

zooid includes the stalk and stolon. I have limited attention in this study to the calyx, because the kamptozoan calyx is the zooidal element most comparable to the reproductive zooids of other colonial animals such as hydroids, bryozoans, and ascidians.

The sexual modes of most barentsiid kamptozoans have not been well characterized. It has been suggested that most or all kamptozoan species are hermaphroditic (Nielson, 1989), and that colony-level hermaphroditism achieved by gonochoric calyces (mode HhG; Fig. 1) is the rule in the genus *Barentsia* (Emschermann, 1985, 1994). The only two studies that have carefully investigated sexual mode in this genus showed that *Barentsia discreta* has mode HhG (Mukai and Makioka, 1980; Emschermann, 1985). I was therefore surprised when my field observations of *Barentsia hildegardae* suggested that it might be gonochoric at the colony level (mode GgG; Fig. 1). I monitored calyces, colonies, and genets of *B. hildegardae* for more than one year to verify that the species was indeed comprehensively gonochoric.

For comparison, I also examined five other barentsiid species. The sexual modes of *Barentsia conferta* and *B. ramosa* were previously unknown; my data suggest that both species are comprehensively gonochoric (mode GgG). The literature regarding the sexual modes of *B. benedeni* (Foettinger, 1887; Ehlers, 1890; Mariscal, 1965; Emschermann, 1994) and *B. discreta* (Ehlers, 1890; Oka, 1895; Marcus, 1939; Mukai and Makioka, 1980; Emschermann, 1985) contains conflicting reports of both comprehensive gonochorism (GgG) and colony hermaphroditism (HhG). My results indicate mode GgG for *B. benedeni*, and mode HhG for *B. discreta*. Finally, my results confirm an earlier report (Johnston and Angel, 1940) of comprehensive hermaphroditism (mode HhH; Fig. 1) for *B. aggregata*.

Materials and Methods

Determining the sexual mode of *Barentsia hildegardae*

Barentsia hildegardae Wasson, 1997, endemic to the northeastern Pacific, is common in the San Juan Archi-

pelago, Washington (Wasson, 1997). I examined a population of *B. hildegardae* growing on the floating dock at the University of Washington's Friday Harbor Laboratories (FHL), San Juan Island, Washington, and identified the sexual mode (G, S, or H) of calyces, colonies, and genets.

This species grows abundantly on solitary ascidians (especially *Pyura haustor*) living on the FHL dock, and each ascidian may be host to many colonies. To assess field patterns of sex distribution, I collected 40 ascidians from the dock between May and July 1993, and removed and sexed all *B. hildegardae* calyces from a single 2-cm² area on each ascidian. Because it was not possible to determine colony boundaries, these 2-cm² plots may have contained multiple colonies.

To monitor known colonies of *B. hildegardae* over time, I grew colonies on glass microscope slides and tracked them for one year. I removed small pieces of colonies from ascidians on the FHL dock and tied them to slides (one fragment per slide). These slides were placed in fenestrated plastic slide boxes; the boxes were enclosed in nylon mesh (knee-high stockings) to reduce fouling, and were hung with ropes (about 2 m long) from the dock. Within a few weeks, stolons had grown and attached these colony fragments to the slides. During April 1993, 130 such slide colonies were started from 20 ascidians. In May 1993, 7 of these colonies originating from 7 ascidians were chosen, and 10 new, replicate slide colonies were created from each one, making a total of 200 slides (the 130 original ones and 70 replicate slides of known genetic origin).

Slides were examined eight times between June 1993 and July 1994, about every 6 to 8 weeks. At each sampling date, slide boxes were brought into the laboratory for 1 week. Each slide was carefully cleaned with a fine paintbrush, and the number of female, male, and immature calyces was noted. The slide boxes were then enclosed in clean nylon stockings and returned to the FHL dock. From July 1994 to August 1995, the slides were left unattended off the floating dock. They were then reexamined in August 1995.

Other barentsiid species

Barentsia conferta Wasson, 1997, and *B. ramosa* (Robertson, 1900) are both endemic to the northeastern Pacific; both of these species are found primarily in the low intertidal zone of exposed coasts (Wasson, 1997). Between May 1991 and July 1995, I collected colonies of *B. conferta* from a rocky shelf and *B. ramosa* from under overhangs in the low intertidal zone at Natural Bridges State Beach, Santa Cruz, California. *B. conferta* grew mainly in dense aggregations on articulated coralline algae; *B. ramosa* grew in dense clumps on rocky overhangs. I examined a total of 48 aggregations (presumed colonies) of *B. conferta* and 33 aggregations of *B. ramosa*, counting male, female, and immature calyces.

Barentsia benedeni (Foettinger, 1887) is a cosmopolitan species, found in bays and harbors around the world (Wasson, 1997). In July 1993, I collected five mussels bearing colonies of *B. benedeni* from floating docks in Lake Merritt, Oakland, California. The calyces in these colonies were not sexually mature. I removed 30 small colony portions from the mussels and grew these fragments on glass slides on a sea table at Long Marine Laboratory, Santa Cruz, California. In February 1994, when 21 of these 30 colonies sexually matured, I examined and sexed all the calyces of each colony. I also examined and sexed a preserved colony of this species from Lake Merritt, in the collection of the California Academy of Sciences (#10510).

Barentsia discreta (Busk, 1886) is widely distributed in most of the world's oceans, in both deep and shallow waters (Wasson, 1997). In October 1994 I obtained two large colonies of *B. discreta* from Mission Bay, San Diego, California. The calyces were not sexually mature. I removed 10 small portions of one colony and 11 of the other and grew them on glass slides on a sea table at Long Marine Laboratory. In August 1995, when the calyces had sexually matured, I examined these 21 slide-grown colonies for the presence of male, female, and immature calyces. I also examined specimens of *B. discreta* in the collection of the Allan Hancock Foundation, University of Southern California. This material (57D/1–10) consisted of 10 whole mounts (slides) of colonies from California, Mexico, Ecuador, and Peru.

Barentsia aggregata Johnston and Angel, 1940, is a little-known species of Antarctic waters. I examined all the specimens of *B. aggregata* deposited in the South Australian Museum, Adelaide, by Johnston and Angel from collections of the BANZ Antarctic Research Expedition (1929–1931) at Macquarie and Heard Islands. This material (E944–946; 948–953; 2132, 2133, and unregistered specimens in the BANZARE collection) consists of 10 preserved (wet) colonies, 9 whole mounts (slides) of colonies, and 31 slides of sectioned calyces.

Results*Barentsia hildegardae*

Every sexually mature calyx of *Barentsia hildegardae* was either male (Fig. 2a) or female (Fig. 2b); a calyx never contained both testes and ovaries. Furthermore, examination of thousands of calyces during the course of this study (in field samples and in slide-grown colonies) never revealed a calyx with ripe ovaries and degenerating testes, or vice versa.

Most 2-cm² plots on ascidians at Friday Harbor contained calyces mainly or entirely of one sex (Fig. 3a), although many plots contained calyces of both sexes. I was unable to determine how many colonies were growing in a single plot. In plots containing both sexes, I never observed male and female calyces growing along the same stolon.

Single colonies grown on slides always consisted of calyces of only one sex. Of the 200 slide colonies sampled eight times over one year, none was ever found with a mix of sexes. Furthermore, none of the 200 slide-grown colonies changed sex over the year (June 1993–July 1994) of monitoring. Although most of the 200 colonies were either dead or nearly so by the end of the second year, 31 colonies were sexually mature in August 1995. All of these were still the same sex as the original colony had been.

Over the yearlong sampling period, all 70 replicate colonies of seven genets remained the same sex as the original colonies they were taken from, regardless of size or age, and regardless of whether they were raised in boxes with colonies of the same or opposite sex. Furthermore, 17 replicate colonies representing four genets were still sexually mature at the end of the second year; all were still the same sex as their source colonies.

Colonies grown on glass slides and kept in jars of seawater in the laboratory under various food, water flow, and temperature levels (as part of growth experiments unrelated to the present study) also contained calyces of only a single sex, and did not change sex over the course of the three-month experiments.

Barentsia conferta, *B. ramosa*, and *B. benedeni*

Calyces of *Barentsia conferta* and *B. ramosa* always contained either testes or ovaries, never both. Aggregations of calyces of these species always consisted mainly or entirely of one sex (Fig. 3b, 3c). In aggregations that contained both sexes, male and female calyces were not randomly distributed. Instead, the aggregation consisted mostly of calyces of one sex, and the calyces of the opposite sex were limited to one patch or one region of the aggregation. Whenever I found an aggregation containing both sexes, I teased it apart and found that the male and

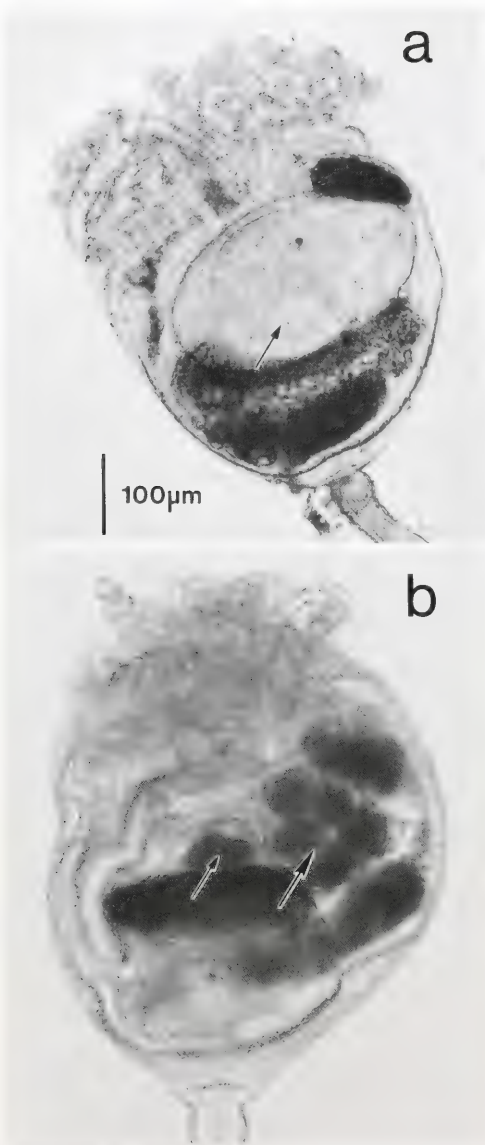


Figure 2. *Barentsia hildegardae*. (a) Male calyx in right-side view; right testis marked by arrow. (b) Female calyx in right-side view; right ovary marked by smaller left arrow; brood chamber containing embryos marked by larger right arrow. Scale bar applies to both a and b.

female calyces were growing on different stolons; I never observed male and female calyces growing on the same stolon.

Calyces of *Barentsia benedeni* contained either ovaries or testes, never both. Each of the 21 slide-grown colonies from Lake Merritt consisted of calyces of a single sex, although colonies did not contain very many sexually mature calyces (Fig. 3d). There were considerably more female than male colonies, probably because I had oversampled a single large female colony when originally removing colony portions from the mussels to generate the slide-grown colonies. The colony at the California Academy of Sciences (#105010) was huge and overwhelmingly female, consisting of about 950 female and 85 immature calyces (no male calyces were present); this colony is represented by the leftmost bar in Figure 3d.

Barentsia discreta

Calyces of *Barentsia discreta* (both museum and slide-grown colonies) contained either testes or ovaries, never both. In June 1995, when the slide-grown colonies first became sexually mature, only male (and immature) calyces were present. In July 1995, a few female calyces were present together with numerous male calyces in the same colonies. In August 1995, 11 of the 21 slide-grown colonies contained both male and female calyces at the same time, while 10 contained only male calyces; no colonies consisted only of female calyces (Fig. 3d). Five colonies in the collection of the Allan Hancock Foundation (57D/1, 3, 7, 8, 10) from California, Mexico, Ecuador, and Peru also had male and female calyces arising from the same stolon within a colony.

Barentsia aggregata

Many calyces in the South Australian Museum's collection of *Barentsia aggregata* from the Antarctic contained both ovaries and testes. Sectioned material (E2132) revealed particularly clearly the simultaneous presence in a single calyx of testes with ripe sperm, ovaries with yolk eggs, and a brood chamber with embryos. Although most preserved colonies consisted of calyces containing both ovaries and testes, a few contained only male or only female calyces.

Discussion

Comprehensive gonochorism (mode GgG): common in the genus Barentsia?

Of all the potential sexual modes for colonial animals (Fig. 1), mode GgG is the most difficult to demonstrate. To eliminate the possibility of sequential hermaphroditism at any level, zooids, colonies, and genets must be monitored over time. To thoroughly exclude genet her-

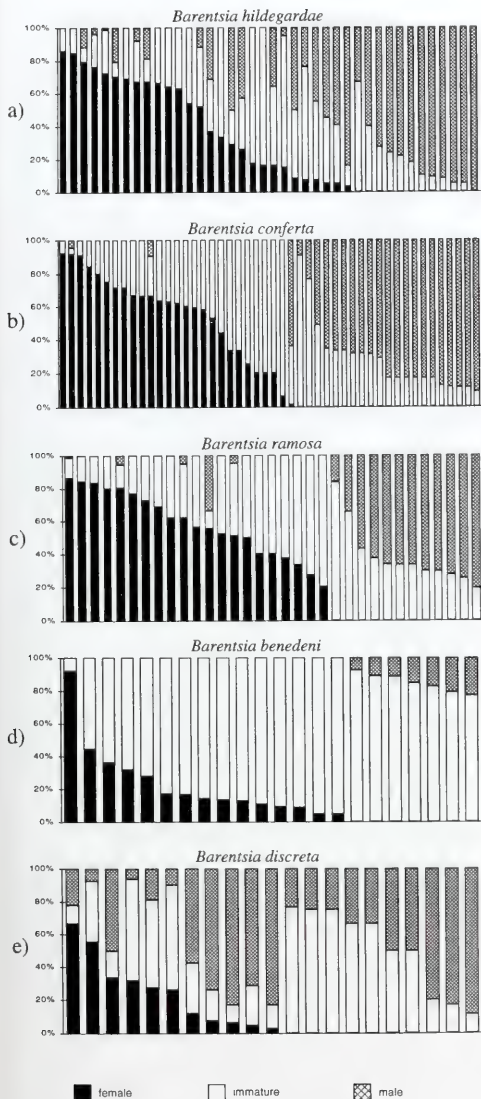


Figure 3. Patterns of sex distribution in five barentsiid kamptozoon species. For each species, all calyces from a plot, aggregation, or slide were sexed microscopically. Percent maturity is shown on the y-axis; plots, aggregations, or slides are shown on the x-axis and have been arranged by decreasing female maturity and increasing male maturity. (a) *Barentsia hildegardae*. Results from 40 (2 cm²) plots on ascidians; a plot contained an average of 38 calyces. (b) *Barentsia conferta*. Results from 48 aggregations on coralline algae; an aggregation contained an

maphroditism, replicate colonies of the same genet must also be examined.

The data for *Barentsia hildegardae* represent the first compelling demonstration of colony and genet gonochorism (mode GgG) in a kamptozoon. Earlier reports of colony gonochorism in the genus (Table I) were less reliable because, based as they were on isolated or brief observations of living colonies or scrutiny of fixed specimens, they could not eliminate the possibility of sequential hermaphroditism at any level. These studies also did not attempt to assess the sexual mode of whole genets.

Barentsia hildegardae evidently displays mode GgG. Calyces contained gonads of only one sex, and did not change sex over time in slide-grown colonies. Every one of the 200 slide-grown colonies contained calyces of only one sex; none changed sex over the one-year sampling period; those that survived through a second year remained the same sex as well. Male or female, the 70 replicate colonies of 7 genets also did not change sex over the entire sampling period.

Although slide-grown colonies of *B. hildegardae* provided unambiguous evidence for comprehensive gonochorism, the samples of colonies growing on ascidians (Fig. 3a) did not. Most of the 2-cm² plots were skewed heavily toward male or female calyces, but many contained a mix of sexes. I conclude that these ascidians hosted multiple colonies of *B. hildegardae* growing intertwined: each 2-cm² plot probably contained calyces mainly from one colony (and thus of one sex), but also contained a few calyces from adjacent colonies (sometimes of the opposite sex). This result highlights the importance of knowing colony boundaries; in this case, field sampling alone could not have distinguished mode GgG from mode HhG.

The more limited data for *Barentsia conferta*, *B. ramosa*, and *B. benedeni* suggest that these species are also comprehensively gonochoric (mode GgG). Field sampling was more strongly suggestive of colony gonochorism for *B. conferta* (Fig. 3b) and *B. ramosa* (Fig. 3c) than for *B. hildegardae* (Fig. 3a). Both *B. conferta* and *B. ramosa* grow more densely than does *B. hildegardae*, thus limiting the potential for intrusion by colonies of different genets. The 21 slide-grown colonies of *B. benedeni* had few sexually mature calyces, but the pattern (Fig. 3d) was consistent with colony gonochorism, as was the huge, wholly female preserved colony that I examined. At the

average of 127 calyces. (c) *Barentsia ramosa*. Results from 33 aggregations on rocky overhangs; an aggregation contained an average of 269 calyces. (d) *Barentsia benedeni*. Results from 21 slide-grown colonies and one large preserved colony (represented by left-most bar); a slide contained an average of 46 zooids; the preserved colony contained 1035 calyces. (e) *Barentsia discreta*. Results from 21 slide-grown colonies; a slide contained an average of 30 calyces.

Table I

Sexual modes reported in the genus *Barentsia*

Sexual mode	Species	Reference
GgG	<i>B. benedeni</i>	Foettinger, 1887; Ehlers, 1890; Mariscal, 1965; Wasson, this paper
	<i>B. conferta</i>	Wasson, this paper
	<i>B. discreta</i>	Oka, 1895; Marcus, 1939
	<i>B. gracilis</i>	Foettinger, 1887; Marcus, 1939
	<i>B. hildegardae</i>	Wasson, this paper
	<i>B. laxa</i>	Marcus, 1939*; Toriumi, 1951*
	<i>B. ramosa</i>	Wasson, this paper
HhG	<i>B. benedeni</i>	Emschermann, 1994
	<i>B. discreta</i>	Ehlers, 1980; Mukai and Makioka, 1980; Emschermann, 1982; 1985; 1994; Wasson, this paper
	<i>B. elongata</i>	Emschermann, 1994
	<i>B. gracilis</i>	Emschermann, 1994
	<i>B. matsushimana</i>	Emschermann, 1982
HhH	<i>B. aggregata</i>	Johnston and Angel, 1940; Wasson, this paper
	<i>B. laxa</i>	Harmer, 1915†

The only reports that are based on repeated examination of the same colonies to determine colony (as well as calyx) sexual mode are those of Mukai and Makioka (1980), Emschermann (1985), and Wasson (this paper); the species examined by these authors are shown in boldface to emphasize that their sexual mode is fairly certain. The sexual modes of the remaining species are less certain, and some appear under two sexual modes in this list. For explanation of the three-letter abbreviations for the sexual modes, see Figure 1.

* The species identified as *Barentsia laxa* by Marcus and Toriumi is probably *B. elongata* (Emschermann, pers. comm.).

† The species identified by Harmer as *Barentsia laxa* is probably undescribed; his material (British Natural History Museum 1916.8.23.38) has distinctly annulated, tall zooids very different from those of *B. laxa*.

calyx level, gonochorism seems certain for all three of these species, and at the colony level, gonochorism seems very likely as well. However, replicate colonies of the same genet were not examined, so genet gonochorism has not been demonstrated. And the same colonies were not observed over time, so the possibility of sequential hermaphroditism cannot be entirely excluded, either.

The sexual modes of *B. conferta* and *B. ramosa* are described for the first time in this paper. There are conflicting reports about the sexual mode of *B. benedeni* (Table I). Foettinger (1887), who described the species, was convinced that colonies as well as calyces are gonochoric, and Ehlers (1890) and Mariscal (1965) supported his view. However, Emschermann (1994) reported colony hermaphroditism via gonochoric calyces of both sexes

(mode HhG) for this species. My observations support the findings of the earlier investigators.

Mode HhG in *Barentsia discreta*

Mode HhG is much easier to demonstrate than mode GgG because the presence of gonochoric calyces of both sexes along the same or clearly connected stolons is the only evidence needed. Replicate colonies of the same genet need not be examined, because a genet consisting of simultaneously hermaphroditic colonies is necessarily simultaneously hermaphroditic itself. However, it is critical that male and female calyces be observed along the same stolon or on stolons whose structural continuity can be demonstrated, or that both sexes be observed in single colonies grown in isolation. Merely observing male and female calyces growing together in a clump does not suffice to identify mode HhG, because such juxtaposition may also result from the intertwined growth of gonochoric colonies (mode GgG) of both sexes, as in field samples of *B. hildegardae* (Fig. 3a). Already in 1890, Ehlers noted how difficult it is to determine the sexual mode of interwoven kamptozoon colonies.

The sexually mature colonies of *Barentsia discreta* clearly displayed mode HhG: gonochoric calyces of both sexes were simultaneously present in the same colonies (Fig. 3d). This contradicts earlier reports (Oka, 1895; Marcus, 1939) of colony gonochorism, but supports the findings of one older report (Ehlers, 1890, on *B. macropus* = *B. discreta*) and two recent, detailed studies (Mukai and Makioka, 1980; Emschermann, 1985) of sexual reproduction in *B. discreta* (Table I). The sex of calyces of *B. discreta* is environmentally determined (Emschermann, 1985), and this sexual lability results in hermaphroditic colonies. The colony-level sexual mode is just the sum of its component parts: a colony built of calyces that have the potential to be either male or female is hermaphroditic (as in *B. discreta*), whereas a colony built of calyces with fixed sex, either male or female, is gonochoric (as in *B. hildegardae* and the other comprehensively gonochoric species).

Emschermann (1982, 1994) has reported mode HhG for four other barentsiid species (Table I).

Simultaneously hermaphroditic calyces: mode (HhH) overlooked

It is quite easy to demonstrate that a species is comprehensively hermaphroditic (mode HhH). Observations of mature ovaries and testes within the same calyx are sufficient. Examination of an entire colony or replicate colonies of the same genet is not required, because simultaneous hermaphroditism of the calyx necessarily implies simultaneous hermaphroditism of both the colony and the genet. It is also not essential to monitor calyces or colo-

nies over time, although that is certainly the best way to observe the timing of maturation and to detect tendencies toward protandry or protogyny.

It has been suggested that all *Barentsia* species have gonochoric calyces (Mariscal, 1975; Emschermann, 1994). Johnston and Angel (1940), however, reported hermaphroditic calyces of *Barentsia aggregata*, and my examination of their material confirms that calyces of this species often contain both mature testes and ovaries. Mode HhH thus is represented in the genus *Barentsia* (Table I).

Although many calyces were simultaneously hermaphroditic, justifying the designation of the species as HhH, some calyces appeared to be only male or only female. Perhaps a period of male maturity precedes simultaneous hermaphroditism, and a period of female maturity follows degeneration of the testes. Or, intriguingly, perhaps sex determination is so labile in this species that fully mature calyces can be either male, female, or hermaphroditic. To distinguish among these alternatives, living colonies must be observed over time.

Harmer (1915) reported hermaphroditic calyces in a kamptozoon from Indonesia that he identified as *Barentsia laxa* (but that almost certainly represents an undescribed species, with tall, flexible, deeply annulated rods). His illustration (pl. 2, fig. 11 in Harmer, 1915) of a hermaphroditic calyx is unconvincing; it appears to be a female with embryos. His material (British Natural History Museum 1916.8.23.38) is in such poor condition that I was not able to see ovaries and testes, so his report remains unconfirmed.

A diversity of sexual modes in the genus Barentsia

The suggestion that most or all species of kamptozoans are hermaphroditic (Nielsen, 1989) has been perpetuated in many invertebrate zoology textbooks (e.g., Brusca and Brusca, 1990; Ruppert and Barnes, 1994). Within the genus *Barentsia*, hermaphroditic colonies with gonochoric calyces (HhG) were considered the rule (Emschermann 1985, 1994). However, the data presented here demonstrate that three sexual modes (GgG, HhG, and HhH) are expressed by species of *Barentsia*, and that comprehensive gonochorism (mode GgG) may be at least as common as HhG. In fact, I hypothesize, on the basis of a recent phylogenetic analysis of northeastern Pacific *Barentsia* species (Wasson, 1997), that comprehensive gonochorism is plesiomorphic for the genus, and hermaphroditism is derived.

What selective pressures might have fostered the evolution or maintenance of such contrasting modes of sex? Comprehensive gonochorism (GgG), as in *Barentsia hildgardae*, prevents the self-fertilization that might occur if calyces or colonies were hermaphroditic. Such forced

outcrossing may be favored under stable environmental conditions and high population densities, where a mate of the opposite sex is always likely to be nearby. Mode HhG, as in *B. discreta*, may combine some advantages of gonochorism (e.g., construction of more specialized reproductive structures) at the level of the calyx with some advantages of simultaneous hermaphroditism (e.g., doubling the chance of encountering a mate) at the level of the genet (Ghiselin, 1969). Furthermore, a strategy of phenotypic sex determination of calyces (as in *B. discreta*'s HhG) may be advantageous in unpredictable environments (Charnov and Bull, 1977) and under variable population densities. For example, a colony might continually adjust its production of male and female calyces in response to environmental cues, including local densities of male and female calyces in neighboring colonies. The calyx hermaphroditism (HhH) of the Antarctic *B. aggregata* may be favored under stringent environmental conditions in which the overall chance of fertilization is very low because the breeding period is short, growth is slow, and population densities are low. Under such conditions, when gametes of the opposite sex are only rarely encountered, calyx hermaphroditism may be a good strategy (Ghiselin, 1969). And simultaneous hermaphroditism may also allow for self-fertilization, if the benefits of any sex at all outweigh the costs of inbreeding. A taxon such as the genus *Barentsia*, in which closely related species have remarkably different sexual modes, would be an excellent model system for testing such hypotheses about the evolution of different colonial modes of sex, by mapping ecological data about environmental conditions, population density, and self-fertilization on a robust phylogenetic tree.

Comparisons with other colonial animals

Colonial cnidarians (hydrozoans and anthozoans) express all three sexual modes (GgG, HhG, and HhH) that barentsiid kamptozoans do (Fadallah, 1983; Fautin, 1992). In addition, some colonial hydrozoans and anthozoans have mode HhS, in which sequentially hermaphroditic calyces are not synchronized in their sex change, so that the colony as a whole is simultaneously hermaphroditic (Wasson and Newberry, 1997). This mode has not yet been reported for a barentsiid kamptozoon.

Bryozoans and colonial hemichordates (pterobranchs) display two of the three modes (HhG, HhH) described for barentsiid kamptozoans, but apparently have no members with comprehensively gonochoric (GgG) colonies (Hadfield, 1975; Reed, 1991). Some bryozoans, like some colonial cnidarians, also exhibit mode HhS.

Colonial urochordates (ascidians and thaliaceans) overwhelmingly display one mode of sex also found in barentsiid kamptozoans, namely HhH (Berrill, 1975). A

very few urochordates reproduce by means of modes GgG, SsS, and HhS (Berrill, 1975).

The sexual mode of many colonial animals is still incompletely known. Many reports describe only the sexual mode of zooids, not of colonies and certainly not of whole genets. Few researchers have observed colonial animals over time, as is necessary to distinguish gonochorism from sequential hermaphroditism at any level; even fewer have looked at multiple colonies of the same genet, as is required to determine the genet's sexual mode. Only as the dynamics of sexual reproduction are thoroughly investigated at all three levels—zooid, colony, and genet—can we begin to understand the phylogenetic distribution of colonial sexual modes and the influence of evolutionary pressures on them.

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Nearly Optimal Foraging in Patches Under Nutrient Constraints

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Abstract. Three feeding strategies with different rules for prey selectivity were evaluated by Monte Carlo computer simulation. The predator must obtain a minimum quantity of each of three different nutrients, and it sequentially encounters one of three kinds of prey that differ in both their nutrient compositions and their relative abundances. Within patches, prey may be randomly dispersed or aggregated with Markovian transition probabilities. Overall cost is the sum of search time plus consumption cost plus emigration (between-patch traverses) risk. The predator will emigrate if it is unsuccessful in acquiring a minimum of Γ units of any needed nutrient within the T most recent prey encounters. The three strategies are (1) no discrimination—with potentially high consumption costs, (2) minimal consumption—with potentially very prolonged search times, and (3) a hybrid strategy that requires a physiological monitoring of net nutrient acquisition (or the bookkeeping of highly correlated gustatory cues). Each strategy has its characteristic dynamics and optima, but collectively these simulations show that no one strategy is superior and that over a large portion of the parameter space the differences in overall cost are relatively small.

Introduction

This study uses computer simulation to examine the consequences of different consumption (or foraging) rules when a predator must obtain a minimum amount of three different nutrients from prey that differ in their nutrient composition and their abundance. Two basic questions are addressed: (1) What kinds of decision rules are useful for a predator with a nutrient-constrained diet, and how

robust are these rules over the parameter space of the model? (2) When prey are distributed in patches (in which one kind of prey predominates), what foraging strategy and which decision rules for leaving nonproductive patches are superior?

This general model can be used for many species of predator, but a particularly apposite example is that of an omnivorous intertidal gastropod, moving randomly over rocks with encrusting or sessile prey in an area where, because of both accident and microgeographic variation, one rock is colonized chiefly by barnacles, another by algae, yet another by mussels, and so on. If individual prey are refractory (like barnacles), the consumption costs may be substantial compared to the search costs. The gastropod emigrates by releasing its hold on the substrate and allowing wave action to redeposit it at some distance from the previous site; here the main cost of emigration could be exposure to predation and the risk that no suitable site whatsoever is found, but these costs can be converted into the currency of "time" commensurate with the search and consumption costs. The omnivorous feeding of intertidal gastropods has been extensively studied (see Largen, 1967, and Vadas *et al.*, 1994, on the dogwhelk, *Nucella* [= *Thais*]; Menge, 1974, on *Acanthina*; Wood, 1968, on the oyster drill, *Urosalpinx*), although direct evidence for nutrient-constrained diets in these species is sparse. However, Speiser and Rowell-Rahier (1993) have found prey selectivity in a helicid snail, *Arion*, a terrestrial relative of intertidal gastropods which shares their proverbial locomotion rates.

In my simulations, the search for suitable prey is a stochastic process (Oaten, 1977; Green, 1987)—hence replication is necessary to evaluate the different factors in the experimental design. This model is intended to be simple but general: simple in that there is no satiation, no contests or competition for resources, and the predator

encounters prospective prey as a consequence of simple Markovian transition probabilities. The model is general in that there are three dietary constraints with three kinds of prey, and two parameters for prey distribution (relative abundance and spatial aggregation).

In the past 20 years, many papers on foraging strategies have been published, and it is not possible to review them all. Pyke *et al.* (1977), Pyke (1984), and Real and Caraco (1986) are good overviews; Pulliam (1974, 1975) succinctly introduces the key points addressed here; and see the Discussion. Many of the recent papers have been based on field studies, which augment the earlier theoretical and laboratory investigations. However, a survey of the literature shows that studies of nutrient-constrained diets seemingly are under-represented (Westoby, 1978; Belovsky, 1990). There are probably two main reasons for this. First, for many species, nutrient considerations are not important—the predator may be essentially monophagous, or if not, it may fulfill all its nutritional needs by serendipitously consuming prey with complementary nutrients without requiring specific adaptations for prey discrimination. Second, it is much more difficult to assay an array of nutrient compositions within prey species and then to demonstrate that the differences in acquisition rates of these nutrients have significant physiological effects on a predator than it is to measure total calories consumed or tabulate search and handling times.

Methods and Experimental Design

In all the simulations described here, there are three nutrients and the forager is required to obtain a minimum amount [MR] of each kind. There are three species, Prey1, Prey2, and Prey3, and every individual of each species has the same total number of nutrient units summed over the three kinds, but the experimental parameter, ξ , determines the composition of the nutrients within each species. Thus we have a **nutrient matrix**, where K designates the mean nutrient level per individual in arbitrary units—in all the simulations the three nutrients are scaled to this common factor:

	Nutrient1	Nutrient2	Nutrient3
Prey1	$(1 + \xi)K$	K	$(1 - \xi)K$
Prey2	$(1 - \xi)K$	$(1 + \xi)K$	K
Prey3	K	$(1 - \xi)K$	$(1 + \xi)K$

The range of ξ is from 0 (all prey have K units of each nutrient) to 1 (each prey type has $2K$ units of one nutrient, and K and 0 of the other two). We also permit ξ to have negative values in the range of 0 to -1 , and this specifies the following matrix:

	Nutrient1	Nutrient2	Nutrient3
Prey1	$(1 + 2 \xi)K$	$(1 - \xi)K$	$(1 - \xi)K$
Prey2	$(1 - \xi)K$	$(1 + 2 \xi)K$	$(1 - \xi)K$
Prey3	$(1 - \xi)K$	$(1 - \xi)K$	$(1 + 2 \xi)K$

This matrix produces a greater nutrient disparity, with the limit of $3K$, 0, 0 units for $\xi = -1$, but as in the first case all the rows and columns sum to the same value ($3K$).

In these simulations $K = 8$ and the minimum requirement [MR] is 240 units of each kind (the scaling is arbitrary). It is clear that for any value of ξ (positive or negative), the required 240 units of each of Nutrient1, Nutrient2, and Nutrient3 can be met if exactly 10 items of each prey type are consumed (*i.e.*, $[10][3K] = 240$), and this total of 30 prey consumed is a minimum value as well (designated $N_{\min}$). It therefore follows that even with large nutrient disparities ($|\xi|$ near 1), if the abundances of the three prey are nearly equal, the forager can expect to fulfill its nutritional requirements without an extended search (though it would be very lucky indeed to find exactly 10 prey of each type in the first 30 random encounters). If one prey species is much more abundant than the other two, the predator faces a greater challenge. For all values of $|\xi| < 1$, it is clear that an exclusive diet of any one prey type can provide the required amount of all three nutrients, but for $|\xi|$ near 1, a large number of prey must be eaten (*e.g.*, if $\xi = +0.9$ or -0.9 , then 300 of the one prey must be eaten). On the other hand, the predator may consume only the minimal number of prey—10 of each type—but if one prey type is rare, then the forager may require very long search times to find the 10 rare prey.

The within-patch abundance distributions are constructed analogously to the nutrient matrices, with δ determining the relative abundance as follows: For $\delta > 0$, $\text{Prey}_C = 0.333(1 + \delta)$; $\text{Prey}_I = 0.333$; $\text{Prey}_R = 0.333(1 - \delta)$. For $\delta < 0$, $\text{Prey}_C = 0.333(1 + 2|\delta|)$; $\text{Prey}_I = 0.333(1 - |\delta|)$; $\text{Prey}_R = 0.333(1 - |\delta|)$. The subscripts C, I, R indicate the common, intermediate and rare prey types within a given patch, with three kinds of patches ($\text{prey}_C = \text{Prey1}$ or Prey2 or Prey3) represented equally. Thus the overall abundance (for any value of δ) of each prey type over the entire ensemble of patches is 0.333. For example, if $\delta = +0.6$, then in one-third of the patches $\text{Prey1} = 53\%$, $\text{Prey2} = 33\%$, and $\text{Prey3} = 13\%$; in another third, $\text{Prey1} = 13\%$, $\text{Prey2} = 53\%$, and $\text{Prey3} = 33\%$; in the remaining third, $\text{Prey1} = 33\%$, $\text{prey2} = 13\%$, and $\text{Prey3} = 53\%$.

These two experimental parameters, ξ and δ , may be varied independently (see Appendix A), but abundance disparity is of no consequence (costs will always be minimal) if all the prey types are nutritionally equivalent (ξ

= 0). With equal abundances ($\delta = 0$ so all $p_i = 1/3$) and marked nutrient disparities, costs will usually be close to minimal as well, but stochastic variation in encounters will occasionally engender markedly higher costs. (This situation would be reversed of course, if the nutrient compositions were random variables and the prey encounters were strictly deterministic.) Therefore, to avoid the proliferation of experimental treatments, the two parameters are usually varied as pairs, with $\xi = \delta = D$ (the disparity index), with $-0.9 \leq D \leq +0.9$.

Studies on intertidal species, as well as many other field studies, have shown that in nature species are usually not distributed independently, but tend to be clumped or aggregated. (More rarely, prey may be overdispersed, but this is a beneficial situation for foragers with incompletely substitutable resources.) If the prey are distributed independently, then random encounters may be simulated by repeatedly generating a pseudo-random variable uniformly distributed on the interval [0,1] and by designating the prey type encountered in conformance to the within-patch abundances $[p_1, p_2, p_3]$. For aggregated prey, the encounter procedure employs the first-order Markov process defined by the following matrix:

	Prey _{1,t+1}	Prey _{2,t+1}	Prey _{3,t+1}
Prey _{1,t}	$(p_1 + R - 1)/R$	p_2/R	p_3/R
Prey _{2,t}	p_1/R	$(p_2 + R - 1)/R$	p_3/R
Prey _{3,t}	p_1/R	p_2/R	$(p_3 + R - 1)/R$

This matrix specifies the probabilities for encountering Prey_j at time $t + 1$ given that Prey_i had been encountered at time t , where R is the **aggregation index**. If $R = 1$ then all rows reduce to the independent trials vector, $[p_1, p_2, p_3]$, but as R increases, the diagonal entries increase and the other probabilities decrease, which of course means that successive encounters of the same prey type become increasingly likely—in other words, the prey are clumped. Regardless of which prey type is initially encountered upon entering a patch, if the predator remains within a patch for a sufficiently long time, the proportion of encounters converges to the vector of the independent trials process.

What kinds of decisions might a forager make when faced with incompletely substitutable resources? First, within a patch, there are two *unconditional* strategies: the forager can either (1) eat every prey item that it encounters—the **TAKE-ALL** strategy or (2) eat only the minimum number of prey (10 of each)—the **SELECTIVE** strategy. There is a cost associated with both finding the prey (**search** costs) and capturing, ingesting, and otherwise handling the prey that the forager decides to pursue and consume (**consumption** costs). Hence the objective of the model forager is to minimize the time needed to find

and consume enough prey to meet the prescribed nutrient vector.

In addition to deciding whether or not to eat a particular prey item, the predator has the option of leaving one patch (for which the abundant prey species is no longer a desirable item of diet) for another (with the hope that, in the new patch, the abundant prey will provide a needed nutrient). Emigration also entails a cost (**traversal** cost), and it is further assumed that the new patch cannot be selectively chosen (*i.e.*, the three kinds of patches are equally likely, and therefore one-third of the time the forager will emigrate to a patch like the one just abandoned).

In the general model, the environment consists of many patches with each of the three prey types being most abundant in one-third of them, so that overall the prey and nutrient availability is concordant with the dietary requirements. In each simulation, all patches have the same disparity indices [$\xi = \delta = D$] and aggregation parameter, R . The predator does not change from its initial strategy, so that the single-patch simulations are the special case of multiple-patch foraging in which the emigration rule is "remain in original patch." What sort of emigration rules might a predator employ? Because emigration costs (or traverse times) are higher than either search or consumption costs, a reasonable rule would delay emigration as long as the current patch is assessed as "productive" by some criterion. Emigration decisions in previous studies as well as this one are almost always based on the following general principle (Charnov, 1976; Oaten, 1977; Regelman, 1986; Valone, 1992): the predator is able to monitor its foraging success, and when its gain fails to attain a minimum level (in many models this is caused by depletion of limited resources) the predator leaves for potentially more productive regions (or switches to an initially inferior prey). A fundamental premise of these experiments is that the predator's sensory system can monitor the gain of each of the three needed nutrients. This model uses two parameters, T and Γ , for the emigration algorithm that is employed by all strategies: *Remain in the current patch if at least Γ units of any needed nutrient have been acquired over the last T encounters, otherwise emigrate.*

This model assumes that each patch is large relative to the predator's search area and that the search is conducted over previously unexplored territory; in other words, the prey are not depleted.

A final consideration is how to weight the three kinds of incurred costs. If search costs are large compared with consumption costs, the decision is simple: the **TAKE-ALL** tactic is superior; if traversal costs are small and the prey aggregated but very disparate in their nutrient composition, then the best strategy is to emigrate as soon as the

forager is confronted with superfluous prey. The most interesting case is where Search Cost (β_S) < Consumption Costs (β_C) < Traversal Cost (β_T). For the general case, the search cost is 1 in arbitrary "time" units and the other two costs are arbitrary multiples of this unit, but this entails a two-dimensional parameter space for the weighting function. To simplify the experimental design, the following assignments are used:

Cost Parameter:	β_S	β_C	β_T
LOW	1	2	4
MEDIUM	1	3	9
HIGH	1	5	25

Cost = β_S (prey encountered) + β_C (prey consumed) + β_T (patches traversed).

The complete model includes all the parameters mentioned above—all applicable to each consumption rule—and this implies a seven-dimensional space generated by the vector [δ , ξ , R , Γ , T , β_C , β_T]; even if the computational limitations were not a factor, it would not be possible to visualize the results for the fully realized model.

In all experiments, the number of replications is 100 (see Appendix B).

Results

Single-patch foraging without aggregation

Although the stochastic results are central to this study, it is useful to begin by comparing the deterministic and stochastic (independent trials) costs, the former serving as a baseline. In the deterministic case, the prey are encountered and consumed within each small time interval in direct proportion to their abundance. The number of prey consumed by a "deterministic" TAKE-ALL predator confined to a single patch is, when both δ and ξ are positive and both are equal to D , equal to

$$\frac{N_{\min}}{1 - \frac{2D^2}{3}} \quad (1)$$

When *either or both* δ and ξ have negative values, the deterministic consumption costs are the same. For the case where $|\delta| = |\xi| \text{ eq } D$, the total prey consumed is

$$N_{\min}/(1 - D^2) \quad (2)$$

For example, if $\delta = \xi = 0.9$, then 66 prey must be eaten $[30/(1 - 2(0.81)/3) = (30/(0.46))]$. For those cases

where either δ or ξ or both = -0.9 , 159 prey $(30/(1 - 0.81))$ are required—a more than fivefold increase over $N_{\min}$.

A forager in stochastic environments where $\delta = 0.9$ and $\xi = -0.9$ consumes an average of 162 prey (*cf.* 159 above), whereas if both parameters = -0.9 , the average rises to 186 prey. Although a predator will occasionally do better with stochastic encounters than with deterministic encounters, over the long run the latter has lower mean costs. A comparison of the stochastic and deterministic case for TAKE-ALL foraging over all combinations δ , $\xi = \pm 0.3, \pm 0.5, \pm 0.7$, and ± 0.9 showed that the number of prey eaten in the stochastic case is greater by 12% (both $\delta, \xi > 0$), 9% ($\delta > 0$; $\xi < 0$), 6% ($\delta < 0$; $\xi > 0$) and 29% (both $\delta, \xi < 0$) than in the corresponding deterministic case.

The SELECTIVE predator always consumes $N_{\min} = 30$ prey but for any pair of δ and ξ , it must encounter more prey than the TAKE-ALL predator, with much larger numbers for extreme disparities. For example, for $D = -0.9$, the average number of prey encountered is 354, a more than tenfold increase over $N_{\min}$. High consumption costs favor the SELECTIVE tactic and low consumption costs favor the TAKE-ALL strategy.

For deterministic encounters where $\delta = \xi = D < 1$, the SELECTIVE (abbreviated SEL) predator encounters $N_{\min}/(1 - |D|)$ prey and consumes $N_{\min}$ prey. The TAKE-ALL (abbreviated T-A) predator encounters and consumes $N_{\min}/(1 - |D|^2)$ prey. For both, Cost = $C_S + C_C = \beta_S \times \text{prey encountered} + \beta_C \times \text{prey consumed}$, and with $\beta_S = 1$:

$$\text{Cost}_{\text{SEL}} = N_{\min}[1/(1 - |D|) + \beta_C] \quad (3)$$

$$\text{Cost}_{\text{T-A}} = N_{\min}[(1 + \beta_C)/(1 - |D|^2)] \quad (4)$$

These costs are the same for the trivial case, $D = 0$, but the two expressions are otherwise quite different. However, in this specific case, the "cross-over" value for β_C , designated β_C^* (for $\beta_C < \beta_C^*$, $\text{Cost}_{\text{T-A}} < \text{Cost}_{\text{SEL}}$; for $\beta_C > \beta_C^*$, $\text{Cost}_{\text{T-A}} > \text{Cost}_{\text{SEL}}$) is

$$\beta_C^* = 1/|D| \quad (5)$$

This simple relation is unexpected because for other combinations of δ and ξ the algebraic expressions are messy. This concise result is partially deceptive, however, for although it shows that for values of D near zero, the TAKE-ALL strategy is superior to the SELECTIVE strategy over a wide range of β_C , a graph of the costs of the two strategies *versus* β_C (Eqs. 3 and 4) for *small* D shows that the two lines are nearly coincident, and therefore the superiority of TAKE-ALL is inconsequential (because both kinds of predator encounter and consume close to $N_{\min}$

prey). What is important is the converse conclusion: if D is greater than 0.5, then *SELECTIVE* will be superior whenever β_c is greater than 2, and for even larger disparity values, the *SELECTIVE* strategy will always be preferred unless the consumption cost is almost as low as the search cost.

Over a range from moderate to extreme disparities in nutrient composition and abundance and with different levels of the Cost parameter, there is no single optimal consumption [*TAKE-ALL* vs. *SELECTIVE*] strategy. For any particular prey and predator species, the nutrient disparity (ξ) among the different prey is likely to be relatively invariant over ecological time, and consumption costs will usually be nearly constant. But certainly the prey abundances (δ) can vary widely (in this sense perhaps β_c rather than β_s should be fixed in the experimental design, but the distinction is arbitrary; moreover, β_c can encompass secondary considerations such as increased predation risk while eating—which would not necessarily be constant over all localities).

Because either the *SELECTIVE* or *TAKE-ALL* rules can incur very high costs depending on δ and ξ and the relative search and consumption costs, these experiments include a third strategy that might reduce the risk of large costs by being a hybrid between the *TAKE-ALL* and *SELECTIVE* rules. This hybrid is the *THRESHOLD* strategy whereby the predator initially adopts a *TAKE-ALL* strategy but, once it has acquired a *threshold* amount of a specific nutrient, it will no longer eat the prey type which is highest in that nutrient. Usually the threshold value is set to $1.25 \cdot MR$. If it were equal to MR (240 units), this third strategy would resemble closely—but not be identical to—the *SELECTIVE* strategy; if much higher, it would resemble the *TAKE-ALL* strategy. An extensive analysis of the deterministic case is not warranted, but to illustrate the analytical impediments, the deterministic expression for the *THRESHOLD* (abbreviated *THR*) strategy where the threshold = MR and $\delta = \xi = D < 0$ (the simplest case algebraically!) is

Prey encountered

$$= N_{mn} \left\{ \frac{8D^2 - |D| + 2}{(1 - |D|)(1 + 2D^2)(2 + |D|)} \right\} \quad (6a)$$

$$\text{Prey consumed} = N_{mn} \left\{ \frac{6D^2 + |D| + 2}{(1 + 2D^2)(2 + |D|)} \right\} \quad (6b)$$

Figure 1 displays the results of the stochastic simulations for the three strategies where $\delta = \xi = D = \pm 0.3, \pm 0.6$, and ± 0.9 with Cost Parameter = Medium ($\beta_c = 3$). Also shown are the average costs for the 5% most-costly replications (i.e., the “worst 5” of the 100 repli-

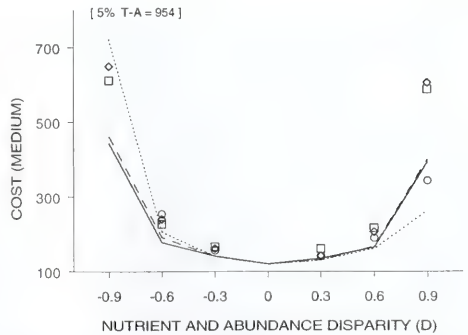


Figure 1. Costs incurred over different prey abundances and nutrient compositions (D) by the three strategies in stochastic single-patch foraging. The lines (dotted = *TAKE-ALL*; solid = *SELECTIVE*; dashed = *THRESHOLD*) connect the mean costs of 10 independent simulations each comprising 100 replicates. The symbols (circles = *TAKE-ALL*; squares = *SELECTIVE*; diamonds = *THRESHOLD*) designate the average of the costs incurred by the 5 replicates with the highest costs (“High 5%”).

cations). Clearly, there is no unconditionally superior strategy.

Prey aggregation

In the analysis above the prey are encountered as an independent trials process; the aggregation parameter, R , alters the probabilities of the transition matrix. Although the stationary vector of the Markov process is the same as the independent trials process, Figure 2 illustrates that clumping with $R = 2$ or $R = 4$ increases the search times with the greatest effect on the *SELECTIVE* and *THRESHOLD* predator. A more detailed comparison between *TAKE-ALL* and *SELECTIVE* is given in Table I; the first entry within each cell is the average cost for the *TAKE-ALL* simulations and the second for *SELECTIVE* simulations. These data show that increasing R has a larger effect on the *SELECTIVE* predator.

Higher R values increase the costs for the single-patch simulations because the greater number of successive encounters with the abundant prey delays the needed acquisition of the scarce nutrients. The mean run-length for prey is equal to $1/(1 - \pi_{ii})$ where the π_{ii} are the diagonal elements of the transition matrix. The mean run-length for the most abundant prey ($i = c$) within a patch (L_c) is, for $\delta > 0$:

$$\text{(Distribution I)} \quad L_c = \frac{3R}{2 - |\delta|} \quad \text{and, for } \delta < 0, \quad (7)$$

$$\text{(Distribution II)} \quad L_c = \frac{3R}{2(1 - |\delta|)} \quad (8)$$

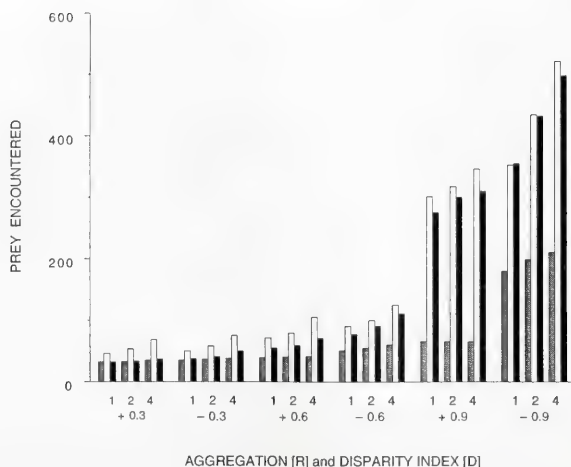


Figure 2. Mean number of prey encountered by predators adopting one of three strategies for single-patch foraging. The data for the different aggregation indices, R , equal to 1, 2, or 4, are nested within the different disparity indices, $D = \pm 0.3, \pm 0.6$, and ± 0.9 . Hatched bars = TAKE-ALL; open bars = SELECTIVE; solid bars = THRESHOLD.

For the rarest prey, for both Distribution I and II,

$$L_R = \frac{3R}{2 + |\delta|} \quad (9)$$

Because $|\delta| < 1$, we have the corresponding ranges for L_C and L_R :

$$\text{I: } 1.5 R \leq L_C < 3 R \quad \text{II: } 1.5 R \leq L_C < \infty$$

$$\text{I, II: } R < L_R \leq 1.5 R$$

From the above inequalities, it is apparent that (a) for any

π_{ii} , the average run length is proportional to R , which can be arbitrarily large; (b) for a given R , the value of δ has little effect on L_R ; and (c) δ has only a modest effect on L_C for Distribution I, but for Distribution II, L_C will attain very high values if $|\delta|$ is near 1 even if R is not especially large. And of course, high values for L_C mean a "long wait" for the scarce prey type.

To encapsulate the results for the single-patch case, I tabulated the costs incurred by the three strategies over 9 combinations of the aggregation index ($R = 1, 2$, and 4) and Cost parameter (Low, Medium, and High) crossed

Table I

Single-patch foraging: Comparison of TAKE-ALL and SELECTIVE strategies over three values of R and six values of the disparity index, D ($n = 100$ for all simulations)

D	Cost Parameter = LOW				Cost Parameter = HIGH			
	R: 1	2	4	Best	1	2	4	Best
+0.3	98 < 106	100 ≈ 114	104 < 128	T-A	195 ≈ 196	199 ≈ 204	208 < 218	T-A
-0.3	106 ≈ 110	110 < 118	115 < 135	T-A	212 > 200	220 > 208	229 ≈ 225	SEL
+0.6	119 < 132	122 < 140	124 < 165	T-A	238 > 222	245 > 230	249 ≈ 255	SEL
-0.6	154 ≈ 150	165 ≈ 160	182 ≈ 185	—	307 > 240	329 > 250	367 > 275	SEL
+0.9	197 < 361	198 < 378	196 < 407	T-A	395 < 451	395 < 468	392 < 497	T-A
-0.9	540 > 414	598 > 495	644 > 582	SEL	1080 > 504	1195 > 585	1268 > 672	SEL

For each comparison, the TAKE-ALL costs are on the left and the SELECTIVE costs are on the right; the Best column indicates the strategy with the lower (or equal) cost for all values of R .

with 6 values of the disparity index, D , giving 56 comparisons. I also included another 27 "worst case" comparisons [using the means of the 5 most costly replications for each strategy] over $D = -0.6, +0.9, -0.9$ and the three R values and the three Cost indices as above. For each comparison, a tie was declared if the average costs of the greatest and least of the three strategies differed by less than 3% (see Appendix B), and if the extremes of the "High 5%" differed by less than 6%.

	Lowest Cost (best strategy)			Highest Cost (worst strategy)		
	Mean	High 5%	Total	Mean	High 5%	Total
TAKE-ALL	18	17	35	17	8	25
SELECTIVE	20	6	26	8	9	17
THRESHOLD	1	3	4	14	9	23

Of the 83 comparisons, the results of 65 are tabulated above; the other 18 were "3-way ties." TAKE-ALL was either the best or the worst in 60 out of the 65 non-tied comparisons. THRESHOLD was in the middle most frequently (59%) and has the lowest best-to-worst ratio (4 "bests" to 23 "worsts").

Multiple-patch foraging

The results for multiple-patch foraging are summarized in Figure 3, constructed analogously to Figure 1. No single pair of values for the emigration parameters (here $\Gamma = 8, T = 4$) can be optimal over all strategies and disparities, but these are "middle-of-the-road" values. A comparison of Figures 1 and 3 clearly shows that when emi-

gration costs are three times greater than consumption costs (and nine times greater than search costs), for values of $|D|$ greater than 0.3, a predator (of any kind) will almost always reduce costs by emigrating.

Because each new patch is chosen as an independent trials process with probability = 0.333, it is easy to show that an average of 4.5 emigrations is required for a predator (of any kind) to visit all three kinds of patches; this is then the optimal number of emigrations for $D < 0$ given that multiple-patch foraging is superior to single-patch. For $D > 0$, emigration to either kind of patch different from the initial patch should suffice, since only one nutrient will be strictly limiting; this requires on the average 1.5 emigrations.

The value of T specifies the physiological memory window, and at one extreme, the emigration decision is determined entirely by the predator's success with the last prey item. For $T = 1$, Γ values of 2 and 8 produced essentially the same emigration rates for both SELECTIVE and THRESHOLD predators; their means are

	$D = +0.6$	-0.6	$+0.9$	-0.9
SELECTIVE:	12.6	11.3	12.6	6.6
THRESHOLD:	5.2	7.8	12.3	7.1

As a consequence of these high emigration rates, the non-emigrant predators had lower costs (both Low and High) except at the highest D values. TAKE-ALL predators tended to remain in the original patch when $\Gamma = 2$, but emigrated when $\Gamma = 8$ (as tabulated below):

TAKE-ALL:	3.0	8.0	3.4	6.6
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All these average emigration rates are higher than the "optimal" values of 1.5 (for $D > 0$) and 4.5 (for $D < 0$).

Table II shows the effect of varying both T (from 1 to 8) and Γ (from 2 to 16) so that all experiments monitor the same rate of nutrient gain (2 units per time interval). The main entries are total cost [Cost Parameter = Medium; $R = 1$] associated with the different pairs of Γ and T . The last row gives the averages over all three strategies and disparities; this is instructive in showing that collectively the differences across the spectrum of short-term ($\Gamma = 2, T = 1$) to longer term ($\Gamma = 16, T = 8$) assessment are not large. Note that greatest and least costs for any strategy are not distributed consistently over either rows or columns, and in a few cases the differences are sizable.

Figure 4 shows the average number of emigrations for each strategy over two values of Γ (4, 16), two values of R (1, 4), and four values of D ($\pm 0.6, \pm 0.9$), where $T = 4$. Note that there is a cluster of points for emigration values from 10 to 12; as was the case for $T = 1$, all

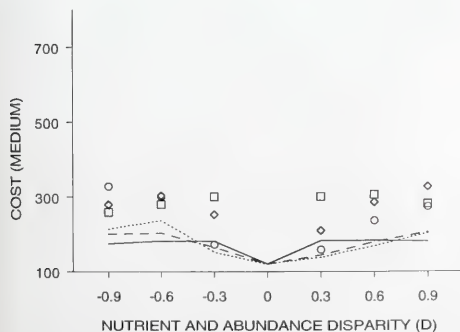


Figure 3. Costs incurred by multiple-patch foragers with $\Gamma = 8, T = 4$; symbols and other parameters—including the 10 repetitions—same as in Figure 1.

Table II

Mean costs (Cost parameter = Medium; $R = 1$) for different emigration parameters (Γ , T) and disparity indices (D)

Strategy	Emigration Parameters [Γ , T]			
	2, 1	4, 2	8, 4	16, 8
$D = +0.6$				
TAKE-ALL	(160)	(162)	(159)	(160)
SELECTIVE	253	199	178	192
THRESHOLD	195	190	180	189
$D = -0.6$				
TAKE-ALL	(205)	(207)	(206)	(207)
SELECTIVE	226	188	187	191
THRESHOLD	224	217	208	201
$D = +0.9$				
TAKE-ALL	204	199	216	232
SELECTIVE	243	201	187	206
THRESHOLD	282	228	217	224
$D = -0.9$				
TAKE-ALL	208	211	218	261
SELECTIVE	195	174	176	186
THRESHOLD	209	199	207	211
Average:	217	198	195	205

Values in parentheses are simulations in which the predator always remained in its initial patch.

three strategies are susceptible to "over-emigration" with consequent increased costs. The outlier is a TAKE-ALL predator that remains in its initial patch because it always obtains at least 4 units of needed nutrient in four encounters.

Table III provides a comprehensive overview of the effect of different Γ values on emigration rates. Note the following points.

1. There are marked differences in the pattern and number of emigrations among the three strategies when viewed as a whole; the average number of emigrations (not including the values for $\Gamma = 32$, which is clearly too stringent a criterion) are TAKE-ALL = 0.84; SELECTIVE = 5.55; THRESHOLD = 3.16.

2. For SELECTIVE and THRESHOLD predators, values of Γ of 1, 2, 4, or 8 usually produce similar emigration rates within each strategy.

3. The aggregation index, R , has only a small influence on the number of emigrations, and the sign of D affects the number in an inconsistent way. For the SELECTIVE and

THRESHOLD predators, the Table III data, excluding those for $\Gamma = 32$, can be synoptically tabulated:

Average Number of Emigrations					
SELECTIVE			THRESHOLD		
	D > 0	D < 0		D > 0	D < 0
R = 1	5.3	4.8	R = 1	2.9	3.2
R = 4	6.5	5.5	R = 4	2.9	3.7

When prey have the more exclusionary nutrient composition ($\xi < 0$), the average number of emigrations is less for the SELECTIVE predator than when the prey are more "substitutable"; the reverse is true for the THRESHOLD predator. The average number of emigrations from Table III for TAKE-ALL ($R = 1$ and $R = 4$ combined) are 0.4 for $D > 0$ and 1.2 for $D < 0$. These are significantly less than the "optimal migration rates" of 1.5 and 4.5, but if only emigrants are averaged, the rates are close to optimal.

4. The SELECTIVE predator has uniformly high emigration rates for all entries with $D = +0.3$ and -0.3 , which represent a quite modest disparity in both abundance and nutrient content

Other considerations

How sensitive is the THRESHOLD strategy to the value of the coefficient that determines the nutritional limits? All simulations reported above used a threshold coefficient of 1.25, and Table IV summarizes the results for other values with $R = 1$, and averages taken over $D = \pm 0.3, \pm 0.6, \pm 0.9$. There is less than a 4% difference in the average costs for the three different coefficients (1.05, 1.25, and 1.5) in both single- and multiple-patch simulations, so its precise value does not seem an important consideration. The simulations employing large values of 2.0 and 5.0 are interesting but extreme; as noted, the data in these columns show congruence to the TAKE-ALL model.

Another consideration is whether one strategy might entail extreme costs more often than another strategy, even when they have similar average costs; many have argued that a predator should choose a strategy that avoids extreme values rather than an alternative that might have significantly lower average costs but is more susceptible to occasional runs of bad luck that engender ruinously high costs (Caraco, 1980; Houston and McNamara, 1985; Real and Caraco, 1986; Weissberg, 1991). In Figure 5, the average costs (of all 100 replications) of the three strategies are plotted against the average of the five replicates with the highest costs ("High 5%"). There are six

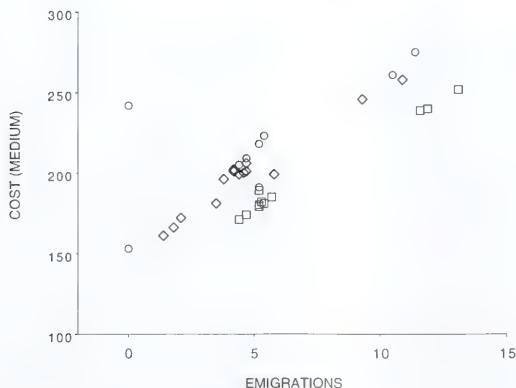


Figure 4. Mean number of emigrations versus average costs for three strategies (symbols same as Figures 1, 3).

points (all for single-patch foragers) that are off-scale; their values and their ratios (5% mean/overall mean) are TAKE-ALL: $490/283 = 1.73$, $1200/877 = 1.37$; SELECTIVE: $1185/425 = 2.79$, $1495/639 = 2.34$; and THRESHOLD: $1384/455 = 3.04$, $1347/640 = 2.10$.

This effect is also seen if the high 5% are substituted for the bottom row ($D = -0.9$) values of Table I that show uniform superiority of SELECTIVE over TAKE-ALL; the values with (*) are exceptions to this pattern.

Cost Parameter	R = 1	R = 2	R = 4
Low	718 > 572	851 < 890*	900 < 1465*
Medium	957 > 602	1135 > 920	1200 < 1495*
High	1435 > 662	1702 > 990	1800 > 1555

These reversals are caused by the higher variances in the SELECTIVE strategy over TAKE-ALL (with THRESHOLD being intermediate). From Tables B1 and B2, the average CVs are

	TAKE-ALL	SELECTIVE	THRESHOLD
single patch:	1.03	1.34	1.18
multiple patch:	1.61	2.04	1.74

The TAKE-ALL strategy has the lowest variance because the predator will always gain some needed nutrient at every encounter, no matter how unfavorably the prey are distributed.

Discussion

Applicability of model

Demonstrating that nutrient constraints are important in shaping feeding patterns is difficult, especially because

there are other reasons for prey selectivity (Westoby, 1978; Speiser and Rowell-Rahier, 1993; Ball, 1994; Vadas *et al.*, 1994; Brown and Morgan, 1995). Nonetheless, there are a number of field studies that support the contention that differences in nutrient composition among prey have ecological and evolutionary significance (Belovsky, 1978 [moose, *Alces*]; Clark, 1980 [feral rat, *Rattus*]; Calvert, 1985 [gorilla, *Gorilla*]; Karasov, 1985 [ground squirrel, *Ammospermophilus*]; Thomson *et al.*, 1987 [junco, *Junco*]; Ritchie, 1988 [ground squirrel, *Spermophilus*]; Wallis de Vries and Schippers, 1994 [cattle, *Bos*]; Forchhammer and Boomsma, 1995 [muskoxen, *Ovibos*]; Kennish, 1996 [crab, *Grapsus*]; see also Wald-bauer and Friedman, 1991 [various insects] and Rapport, 1980 [ciliate protozoan, *Stentor*]).

How relevant are the results of these simulations to the interpretation of such field studies, and how limiting are the assumptions of the models employed? Is this model realistic in requiring a maximum of only three nutrients, in its parameterization of the prey distribution, and in its assumption that a forager can monitor its nutrient acquisition?

1. A comprehensive model of nutrient-constrained foraging should normally require no more than three nutrients (and therefore no more than three prey). More than three nutrients may be desirable in a diet, but deficiencies in the additional nutrients are unlikely to produce strong selective pressures. I could find no evidence that a species in the wild is constrained by more than three nutrients. Calvert (1985) measured 11 constituents in 27 species of plants eaten by the mountain gorilla and found that only three components (lignin, fiber, and protein) determined prey selectivity.

Table III

Average number of emigrations for different values of Γ for $T = 4$ over 6 values of the disparity index, D

		D =	+0.3	-0.3	+0.6	-0.6	+0.9	0.9	+0.3	-0.3	+0.6	-0.6	+0.9	-0.9	+0.3	-0.3	+0.6	-0.6	+0.9	-0.9	
Γ	R	TAKE-ALL								SELECTIVE						THRESHOLD					
		1	1.5	4.3	4.8	10.1	7.7	11.3	16.1	19.6	17.6	13.3	20.2	12.1	1.4	4.8	5.3	9.6	7.2	11.1	
32	1	1.5	4.3	4.8	10.1	7.7	11.3	16.1	19.6	17.6	13.3	20.2	12.1	1.4	4.8	5.3	9.6	7.2	11.1		
	4	1.9	5.2	3.3	10.5	5.7	11.4	21.6	21.6	20.0	13.1	21.8	11.9	2.1	5.4	4.1	11.0	6.0	10.5		
16	1	0	0	0.5	4.3	2.5	4.4	6.2	8.7	11.1	5.6	12.2	4.6	0	1.0	4.3	4.4	2.0	4.4		
	4	0	0	1.1	4.4	2.4	4.7	11.9	11.6	10.8	5.2	11.2	4.7	0.7	3.5	6.2	4.5	7.0	4.6		
8	1	0	0	0	0	1.6	4.4	3.4	2.9	5.1	5.0	4.8	4.5	0	0.4	2.0	3.9	5.0	4.7		
	4	0	0	0	0	1.7	5.2	5.6	5.7	5.1	5.2	6.3	5.2	0.6	2.1	3.1	4.2	4.1	4.7		
4	1	0	0	0	0	1.6	4.6	2.2	3.6	5.2	5.2	5.0	4.6	0	0.3	2.6	5.0	4.4	4.7		
	4	0	0	0	0	1.6	5.4	4.8	5.3	5.0	5.4	5.2	5.3	0.4	1.4	2.5	4.7	4.1	4.6		
2	1	0	0	0	0	0	0	3.1	3.2	5.3	5.4	4.5	4.7	0	0.2	2.7	4.6	4.3	4.6		
	4	0	0	0	0	0	0	5.3	5.3	5.3	5.2	5.1	4.4	0.6	1.8	2.3	3.8	4.0	4.7		
1	1	0	0	0	0	0	0	2.6	3.3	4.8	5.4	4.8	4.8	0	0.4	2.1	4.1	4.5	4.4		
	4	0	0	0	0	0	0	5.5	4.8	5.0	5.2	6.0	4.6	0.6	2.2	3.0	3.9	4.1	5.2		

2. Many prey species may have more than two levels of spatial distribution and abundance, but the slight gain in realism achieved by incorporating a third level would be offset by increased complexity in the experimental design (see Underwood and Chapman, 1996).

3. The nutrient (ξ) and abundance (δ) disparity parameters were subsumed under one disparity parameter, D , and Appendix A considers the consequences of reducing the parameter-space. All nutrients and prey types have the same values in this symmetric model (240 units of each nutrient can be obtained from 10 prey of each type). But these are essentially relative values; the same general conclusions would obtain if different nutrients had differ-

ent minimum requirements and if prey differed in size. Obviously this model loses relevance for extreme departures from this idealized situation—for example, the case where eating a single individual of one kind of prey sufficed for one nutrient, and several hundreds of smaller individuals of a second kind of prey were needed for a second nutrient. If applied to a specific field study, this model could incorporate additional parameters reflecting the particular physiology and ecology of the predator.

4. To an observer, the TAKE-ALL predator behaves within a patch as if it were not subject to any nutrient constraint, and the only evidence that it is monitoring nutrient gain would be its sporadic emigration. In this sense, the TAKE-ALL rule serves as a control to which the SELECTIVE and THRESHOLD strategies may be compared. The simulations assume that the THRESHOLD (and the TAKE-ALL) foragers can monitor nutrient acquisition, whereas the SELECTIVE predators are presumed to have an instinctive prey selectivity shaped by experience over evolutionary time. If individual prey differ in size (or for other reasons differ in nutrient content), SELECTIVE predators, going strictly by the numbers of prey consumed, will be more likely to have nutrient deficiencies than will the predators that can assess nutrient gain. For the THRESHOLD predator, what is the biological justification for the threshold coefficient (here 1.25), which may be either (1) a value crafted by adaptation to allow for imprecision in assessment or inherent variability in nutrient requirements, or (2) a parameter reflecting the time lag between the time of ingestion and the physiological processing of food's nutrient composition (so that some "overshoot" inevitably occurs)? Although nutrient gains usually cannot be assessed immediately in terms of physiological benefit, it is plausible that a species might evolve

Table IV

THRESHOLD strategy with different threshold-limit coefficients: all entries are averages over six values of the Disparity index [$D = \pm 0.3, \pm 0.6$, and ± 0.9]; aggregation index (R) = 1

	THRESHOLD Coefficient				
	1.05	1.25	1.50	2.00	5.00
Single-Patch ($\Gamma = 0$)					
Average prey consumed	34.0	36.1	38.3	41.6 ^a	50.0 ^b
Mean cost [medium]	256	247	249	240	242
$\Gamma = 8, T = 4$					
Average prey consumed	34.0	36.5	39.2	43.4 ^a	56.5 ^b
Average emigrations	3.4	2.8	2.3	1.6 ^a	0.07 ^b
Mean cost [medium]	178	181	184	193	235

^a For both single-patch and $\Gamma = 8, T = 4$, THRESHOLD was equivalent to TAKE-ALL for $D = \pm 0.3$ and $+0.6$.

^b THRESHOLD same as TAKE-ALL for all D except $D = -0.9$.

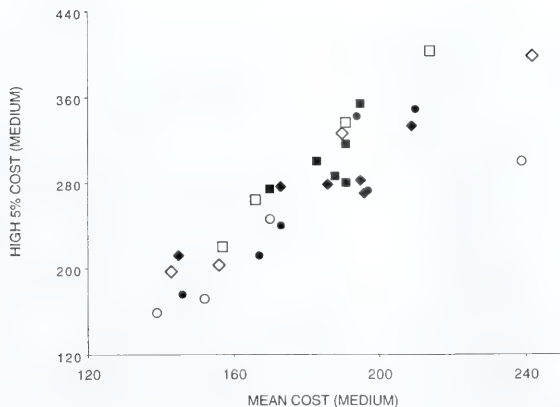


Figure 5. Mean costs versus "High 5%" for the three strategies. $R = 4$; $T = 8$; $T = 4$. Symbols same as Figures 1, 3, with open symbols = single-patch and solid symbols = multiple-patch. $D = \pm 0.3$, ± 0.6 , and ± 0.9 (multiple-patch only).

the capability to monitor nutrient intake more promptly but indirectly by relying on correlated gustatory or olfactory cues.

In light of the above contention, it is helpful to summarize a series of laboratory experiments performed over a number of years using rats subjected to suboptimal diets. Richter *et al.* (1938) showed that laboratory rats given the opportunity to select from a variety of foods (olive oil, casein, sucrose, cod liver oil, wheat germ oil, yeast, and four inorganic salts—all supplied in excess) had the same weight-gain, longevity, and fecundity as those raised on the best commercial diets, while actually consuming a lower total weight of food. In the authors' words, the rats had a "special appetite not only for salt and sugar, but for protein, carbohydrate, sodium, calcium, phosphorus, potassium and . . . [vitamins]." Morrison (1974) demonstrated that well-fed rats typically demonstrated a preference for novelty in their diets, even for substances that ranked very low in palatability (as ranked in prior studies in which alternatives were supplied *ad libitum*).

Rodgers and Rozin (1966) demonstrated that thiamine-deficient rats sought novelty in their diets at a higher level than nondeficient rats, even though previous dichotomous choice experiments had shown that thiamine-deficient rats were unable to detect (or at least did not prefer) a thiamine-supplemented diet item over one lacking in thiamine. Rodgers (1967) concluded that "there are a rather large number of needed vitamins and minerals, and it is unreasonable to attribute to the animal the ability to recognize the taste or smell of all of these, as would be required if all specific hungers [his term for prey selectiv-

ity] were unlearned." Consequently, his diet-choice experiments suggest that an inherent bias for novelty in the diet is enhanced when animals are lacking in one or more important nutritional components.

Rozin (1969) has postulated that the preference for novelty is actually an aversion to the food previously consumed, which the rat perceives as being responsible for its decline in vigor. His results corroborated earlier work on rats with vitamin B-complex deficiencies. Harris *et al.* (1933) showed that rats could not detect which one of six foods contained the necessary vitamin supplement, but characteristically ate one or two different alternatives on successive days, with all six eventually being consumed in roughly the same amounts. They concluded that "the ability of the vitamin B-depleted rat to discriminate between diets containing the vitamin and those deficient in it depends not on vague instinct but on an association between the distinctive character of the diet . . . and an experience of prompt beneficial gains . . . which follow immediately [!] on its consumption."

5. This model does not take into account predator satiation, prey depletion, or time constraints (Stephens and Krebs, 1986; Brown and Mitchell, 1989; Ward, 1993b). Satiation imposes an upper limit on a TAKE-ALL predator, and time constraints would limit the number of prey encounters for SELECTIVE and THRESHOLD. A satiated TAKE-ALL predator is likely to behave like a THRESHOLD predator; a SELECTIVE (or THRESHOLD) predator will probably become less discriminating if its foraging time is frequently restricted by extrinsic factors. Species typically subjected to rigorous environmental fluctuations may

have to substitute local [sub]optimal solutions for the globally optimal one (Ward, 1993a, b); his example is oystercatchers, whose prey show intertidal zonation and therefore prey choice is adversely curtailed by tidal cycles. Prey depletion within patches can be accommodated within this model because rate of nutrient gain will necessarily decrease as prey are depleted, triggering emigration as specified by T and Γ (see Brown and Mitchell, 1989; Mitchell, 1990). In sum, the limitations of this model are ones suggested by common sense. If a TAKE-ALL predator typically consumes between 35 and 50 prey, it is unlikely that it could eat 150 to 200 or more prey within a comparable time period when faced with an extremely disadvantageous prey distribution. In this case the outcomes might be reduced fitness, or reversion to an escape behavior such as diapause or migration, or a change in feeding strategy (the TAKE-ALL predator imitates a SELECTIVE one). It is also reasonable to suppose that a SELECTIVE predator, despite its instinct to eat a fixed [small] number of prey, would eventually eat previously rejected prey after extended searches failed to find the few rare prey that minimized consumption costs.

Conclusions

These simulations show that the three foraging strategies are relatively insensitive to variation in T and Γ (Tables II and III), provided neither value is very small or very large. However, the strategies have different optima for these values, and therefore each kind of predator would be likely to adopt different T and Γ values—since these are not fixed by the environment, unlike δ , ξ , and R (see Green, 1987). For example, a TAKE-ALL predator would be expected to employ a higher Γ value than a SELECTIVE one.

Both R and δ are likely to vary over an organism's feeding season and, more importantly, δ (and probably R) will be different in successive patches visited by each predator. These simulations had fixed (within an experiment) values for δ , ξ , and R , but overall the strategies were evaluated over a range of δ (as D) values. In the field, patch sizes will vary, but in this model prey are not depleted, so all patches are large relative to their diminution by consumption. Collectively, these simulations show that T , Γ are generally robust over a range of relative prey abundances; that is, a particular pair of values for T and Γ that are appropriate for habitats with high abundance disparities will adequately serve the predator in low-disparity environments.

The consequences of increased prey aggregation (higher R) are dichotomous: Figure 2 and Table I show that higher R values consistently increase costs for predators confined to just one patch, especially for SELECTIVE

foragers; with emigration, however, higher R values may shorten the time needed to acquire just the one needed prey. If very high R values are common, a species should decrease its memory window (T), thereby expediting emigration from patches that are no longer productive. As pointed out earlier, Table III shows relatively small effect of different R when $T = 4$; but when $T = 1$ ($\Gamma = 8$; Cost factor = Medium), a comparison of costs (over six values of D) for all three strategies reveals that the total costs for $R = 1$ are greater than for $R = 4$ in 15 cases, and are less in only 3. Moreover, a patch in which the prey have a low δ in conjunction with a large R may mimic a patch with much higher δ and no aggregation (independent encounters). Although there are exceptions, the general conclusion is that the deleterious effect of high R on single-patch foraging becomes a mild advantage for multiple-patch foraging.

There are two primary considerations in evaluating the different strategies. (1) How consistently does a strategy minimize costs within patches. (2) How robust is a strategy in keeping emigrations at a close-to-optimum number for given Γ , T over a range of environmentally determined values for δ , ξ , and R .

The quantitative results support the intuitive generalization that for all three strategies, predators should remain in the initial patch if all three prey are common, and should emigrate promptly from patches with high disparities once they have exploited the most abundant prey. The analysis may be extended by the following consideration: for any strategy with given δ , ξ , and β_T , the predator should either remain in the original patch and incur no traversal costs, or should emigrate to successive patches without modifying the emigration criteria, because the patch transition probabilities are independent of previous patches visited (see McNamara *et al.*, 1993). If emigration to a new patch has an expectation of lower overall costs than remaining in the original patch, then Γ and T should depend only on which strategy is employed, not the on values of δ and R . A predator is unlikely to find markedly different values of ξ from patch to patch (but nutrient compositions could vary over locality and season).

For single-patch foraging, with high D values and moderate to large search costs, SELECTIVE is superior to TAKE-ALL. The THRESHOLD strategy, although almost always second-best by a small margin to either SELECTIVE or TAKE-ALL, seems to be a workable compromise (see Figures 1 and 2).

For multiple-patch foraging, from an analysis of the cases for $D = +1.0$ or -1.0 , we can generalize to cases of intermediate disparities. Consider first the case for $D = -1.0$ (each patch is monotypic with completely exclusionary nutrient constitutions). The number of prey en-

countered and eaten for TAKE-ALL will be $30 + 4.5T$, and for SELECTIVE will be 30 eaten and $30.0 + 4.5T$ encountered. The THRESHOLD will be similar to TAKE-ALL. Emigration is a necessity and occurs promptly; the costs are therefore lower than for cases with intermediate values of D.

For the case in which $D = +1.0$, the predator needs to exploit only two different patches. The deterministic TAKE-ALL predator will encounter and eat $39.4 + 1.5T$ prey. The deterministic SELECTIVE predator will eat 30 prey while encountering $52.5 + 1.5T$ prey (and the THRESHOLD predator will be intermediate).

For negative D in Table III, the average number of emigrations is close to 4.5 as theory predicts, but for positive D, the values are usually higher than 1.5. This is a consequence of the fact that even if the second patch is favorable (its most abundant prey is highest in the needed nutrient), there is a probability of $(2 - D)/6$ that the predator will initially encounter superfluous prey and then "mistakenly" emigrate from the patch.

When emigration is an option, for all three strategies over all combinations of parameters (including Γ and T), the total prey encountered is within the range of 30 to 48. This is an important point that supports the conclusion that nearly optimal foraging can be obtained with more than one foraging rule. Consider two cases for multiple patch foraging:

I. Immediate Emigration ($T = 1$; $\Gamma = 8$; these values specify that the predator must emigrate if it fails to acquire at each encounter at least one needed nutrient at a rate equal to its average value over all prey types). These means are taken over all $D (\pm 0.3, \pm 0.6, \pm 0.9)$:

TAKE-ALL: 43.9 eaten and encountered
 SELECTIVE: 30 eaten and 43 encountered
 THRESHOLD: 39 eaten and 44.5 encountered

The TAKE-ALL predator encounters more prey than does the SELECTIVE predator, but emigrates less often, which can offset the apparent inferiority of the TAKE-ALL strategy seen above (e.g., for $D = \pm 0.9$, the average number of emigrations is 5.0 for TAKE-ALL and 9.7 for SELECTIVE).

II. Moderate Time Window ($T = 4$); the means are taken over $\Gamma = 16, 8, 4$, and over all six values of D:

TAKE-ALL: 41.6 eaten and encountered (min = 32.5; max = 52.0)
 SELECTIVE: 30 eaten and 45.5 encountered (min = 39.5; max = 49.1)
 THRESHOLD: 36.5 eaten (min = 32.5; max = 39.5) and 45.7 encountered (min = 32.5; max = 55.5)

The minimum and maximum values for consumption and encounters are also indicated above. The THRESHOLD predator seems inferior to SELECTIVE, but the former has markedly lower average emigration rates (THRESHOLD = 3.2; SELECTIVE = 5.6).

The THRESHOLD strategy seems to be an effective and feasible (that is, biologically obtainable) tactic (Figs. 3, 4, and 5). The extent to which animals in the wild have adopted this "rarely optimal but often nearly optimal" strategy (see Real and Caraco, 1986; Ball, 1994; Ward, 1992, 1993a, b) remains an open question. Given the inherent stochasticity of the foraging process and the fact that throughout much of the parameter space the three foraging strategies dictate similar behavior, this question may be difficult to answer.

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Appendix A

In all the experiments, δ and ξ have the same value ($-0.9 \leq D \leq +0.9$). Although the nutrient and abundance matrices are mathematically equivalent, in the stochastic case, a given nutrient disparity is more costly to the predator than a comparable abundance disparity. For example,

Table A

The effect of different nutrient [ξ] and abundance [δ] disparities, $\Gamma = 8$; $T = 4$; the main entries are the means of (1) prey consumed, (2) prey encountered, and (3) emigrations

	$\delta, \xi =$ +0.9, +0.9	$\delta, \xi =$ +0.9, -0.9	$\delta, \xi =$ -0.9, +0.9	$\delta, \xi =$ -0.9, -0.9
Strategy				
R = 1				
TAKE-ALL	51, 51, 1.6	54, 54, 1.5	49, 49, 4.9	43, 43, 4.4
SELECTIVE	30, 49, 4.5	30, 49, 5.0	30, 42, 4.7	30, 42, 4.5
THRESHOLD	40, 56, 5.0	42, 54, 4.6	37, 56, 5.3	37, 50, 4.7
R = 4				
TAKE-ALL	47, 47, 1.7	53, 53, 1.5	44, 44, 5.5	43, 43, 5.2
SELECTIVE	30, 45, 6.3	30, 42, 5.1	30, 43, 5.1	30, 42, 5.2
THRESHOLD	39, 48, 4.7	41, 51, 4.2	36, 49, 4.9	37, 48, 4.7

if $\delta = -0.3$ and $\xi = -0.7$, a TAKE-ALL predator (in a single patch) will consume 45.0 prey; if $\delta = -0.7$ and $\xi = -0.3$, then only 40.5 prey will be eaten.

In 24 such comparisons where $|\delta| \neq |\xi|$, 19 of these had greater costs when $|\xi| > |\delta|$, and the remaining five were ties. This general pattern also applies to the SELECTIVE and THRESHOLD predators. This is a consequence of the fact that if there is marked nutrient disparity but equal abundances, the predator is still subject to stochastic variation in encounters, whereas if abundances are disparate but prey are equivalent, there will be no deviation from the minimum number.

For multiple-patch foraging, Table A displays data for two additional cases, where $\delta = -\xi = \pm 0.9$. Overall, the data in the inner columns are similar to those in the outer columns for which $\delta = \xi = +0.9$ and $\delta = \xi = -0.9$. The small differences confirm that the nutrient disparity is more costly than an equivalent abundance disparity.

In conclusion, the restriction of the parameter space for spatial distribution and nutrient composition to those values of $\delta = \xi = \pm 0.3, \pm 0.6, \pm 0.9$ does not compromise the generality of the interpretations.

Appendix B

The data in this paper are averages of 100 replicate runs—each starting with a unique seed. Because of the variability from one replicate to another (which is the reason that the worst 5 of 100 replicates have much greater costs than the overall average), it is important to estimate the intrinsic variability between “runs.” Tables B1 (for single-patch) and B2 (for multiple-patch) give the results of 10 repetitions of the “ $n = 100$ ” simulations.

These tables provide the basis for judging whether different parameter sets give significantly different results. The means ($\bar{x}$) from the 10 replicate simulations have a t -distribution with 9 degrees of freedom, with standard deviation s . The 5% significance level for the difference of the means is equal to 2.2 (t with 18 degrees of freedom) times the s value: *i.e.*, if $\bar{x}_1 - \bar{x}_2$ is $> 2.2s$, then the means are significantly different. For most of the simulations, s is roughly proportional to the means; therefore, if two means differ by more than 3% (average CV approximately 1.2 for single-patch foraging) or 6% (average CV approximately 1.8 for multiple-patch foraging), the two costs are considered to be significantly different.

Table B1

SINGLE-PATCH: Means, standard deviations, and coefficients of variation (in %) of foraging costs (Cost parameter = Medium) for 10 replicate simulations, each consisting of 100 independent trials; $R = 1$

D	Sample*	TAKE-ALL			SELECTIVE			THRESHOLD		
		Mean	Std. dev.	CV	Mean	Std. dev.	CV	Mean	Std. dev.	CV
+0.6	All	161.0	1.49	0.93	165.3	2.91	1.76	166.6	1.43	0.86
	5%	188.8	3.39	1.80	215.5	5.82	2.70	209.7	5.23	2.49
-0.6	All	206.7	1.25	0.60	178.2	2.53	1.42	191.3	2.67	1.40
	5%	253.7	4.95	1.95	227.2	7.32	3.22	239.5	6.04	2.52
+0.9	All	263.8	4.54	1.72	392.7	6.41	1.63	400.0	7.12	1.78
	5%	342.5	7.76	2.27	588.0	16.34	2.78	604.8	15.84	2.62
-0.9	All	720.6	6.19	0.86	443.2	2.44	0.55	462.4	3.06	0.66
	5%	939.9	22.04	2.34	611.6	17.65	2.89	648.9	22.00	3.39

* All designates data for all 100 trials; 5% the data for the 5 “most costly” trials.

Table B2

MULTIPLE-PATCH: Means, standard deviations, and coefficients of variation (in %) of foraging costs (Cost parameter = Medium) for 10 replicate simulations, each consisting of 100 independent trials; T = 8; T = 4; R = 4

D	Sample*	TAKE-ALL			SELECTIVE			THRESHOLD		
		Mean	Std. dev.	CV	Mean	Std. dev.	CV	Mean	Std. dev.	CV
+0.6	All	169.1	3.11	1.84	183.5	6.15	3.35	179.5	3.81	2.12
	5%	236.9	9.04	3.82	307.0	27.81	9.06	286.0	11.78	4.12
-0.6	All	236.3	5.08	2.15	181.9	4.04	2.22	203.0	3.92	1.93
	5%	300.0	0	0	280.0	19.11	6.83	303.1	18.70	6.17
+0.9	All	202.6	1.90	0.94	180.8	2.44	1.35	207.0	3.50	1.69
	5%	274.0	11.35	4.14	281.9	14.75	5.23	328.3	22.93	6.98
-0.9	All	214.2	3.26	1.52	174.6	2.17	1.24	200.0	2.45	1.23
	5%	328.1	15.14	4.61	259.3	11.21	4.32	278.5	10.48	3.76

* All designates data for all 100 trials; 5% the data for the 5 "most costly" trials.

Zoogeographic Distributions of the Sibling Species *Mytilus galloprovincialis* and *M. trossulus* (Bivalvia: Mytilidae) and Their Hybrids in the North Pacific

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Abstract. Diagnostic length differences in a PCR amplified fragment of the gene for byssal adhesive protein were used to study the zoogeographic distribution of *Mytilus galloprovincialis* and *M. trossulus* along the west coast of North America and in Japan. The distributions of *M. galloprovincialis* and *M. trossulus* are patchy, although an overall geographic pattern emerges. *M. galloprovincialis* was the only species found on either Kyushu or Honshu, and it was the most abundant mussel from Tomales Bay to San Diego, California. *M. trossulus* was the only bay mussel found on Hokkaido and in Alaska, and it was by far the most abundant mussel along the coasts of Washington and Oregon.

Mytilus galloprovincialis and *M. trossulus* are sympatric and hybridize near Whidbey Island, Washington, in San Francisco Bay, and in San Diego Bay. A second diagnostic anonymous nuclear PCR marker was used to examine the extent of hybridization at Palo Alto, California. At this site, genotypes appeared to be a mixture of *M. galloprovincialis*, F₁ hybrids between *M. galloprovincialis* and *M. trossulus*, and backcrosses between the F₁'s and *M. galloprovincialis*.

The discontinuity between the zoogeographic distributions of these two species at about 40°–41°N latitude in both the eastern and western Pacific suggests that temperature is a factor in determining their present distribution and limiting their dispersal to other regions.

Introduction

Biochemical and molecular systematics have dramatically altered our understanding of the ecology, evolution,

and biogeography of bay or blue mussels of the genus *Mytilus* (McDonald and Koehn, 1988; Koehn, 1991; Seed, 1992; Rawson and Hilbish, 1995). The taxonomy of the *Mytilus edulis* complex has been confused (see Seed, 1992, for a full discussion of species definitions in this genus) as a result of considerable morphological similarity among species. For example, each species varies considerably in size and shape both within and among populations (Seed, 1968). Today, multivariate analyses of morphological shape (McDonald *et al.*, 1991) and analyses of molecular genetic variation (see below) have led to the recognition of three sibling species: *M. edulis* Linnaeus 1758, *M. galloprovincialis* Lamarck 1819, and *M. trossulus* Gould 1850. Early work suggested a simple geographic distribution in the northern hemisphere, with *M. edulis* being found in the temperate regions of the Atlantic and Pacific Oceans, and *M. galloprovincialis* restricted to the Mediterranean Sea. General recognition of *M. trossulus* resulted from surveys of allozyme variation in populations at higher latitudes in both the Atlantic and Pacific Oceans (Koehn *et al.*, 1984; McDonald and Koehn, 1988; Varvio *et al.*, 1988).

The discovery of hybridization among members of the *Mytilus edulis* complex blurred the tidy distribution patterns mentioned above. The first indication that the distributions were more complex than initially proposed was provided by electrophoretic surveys of genetic variation of mussels in the British Isles (Skibinski and Beardmore, 1979; Skibinski, 1983; Koehn, 1991, p. 134), which revealed the presence of *M. galloprovincialis*, and hybridization between *M. edulis* and *M. galloprovincialis* (Skibinski and Beardmore, 1979; Gosling and Wilkins, 1981; Skibinski, 1983; Skibinski *et al.*, 1978; 1980; 1983). Stud-

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ies also revealed that the bay mussels on the Pacific coast of North America were not *M. edulis*, nor were they a single species. McDonald and Koehn (1988), using allozyme markers, identified *M. galloprovincialis* in southern California, *M. trossulus* in northern California and further north, with a hybrid zone in and around San Francisco Bay. This pattern has been verified with studies using markers from mtDNA (Geller, 1994; Geller *et al.*, 1994; Rawson and Hilbish, 1995).

Complexity and patchiness in the distribution patterns of *Mytilus* species have been found not only on a broad geographic scale but also on a microgeographic scale. For example, ecological genetic studies have revealed variation associated with environmental conditions. In the British Isles, the abundances of *M. edulis* and *M. galloprovincialis* vary with both salinity and wave exposure: *M. galloprovincialis* is relatively more common in areas with high salinity and high wave exposure (Skibinski *et al.*, 1983). In a hybrid zone at the salinity gradient between the North Sea and the Baltic Sea, *M. edulis* alleles give way to *M. trossulus* alleles as salinity decreases (Väinölä and Hvilson, 1991).

In the past, taxonomic work on *Mytilus* was made difficult by the lack of an easy and complete means of identifying species. Although identification of *M. edulis*, *M. galloprovincialis*, and *M. trossulus* is possible with electrophoretic surveys of proteins (Koehn *et al.*, 1984; Grant and Cherry, 1985; McDonald and Koehn, 1988; Väinölä and Hvilson, 1991), it is now easier to identify species and their hybrids with DNA markers (Geller and Powers, 1994; Geller *et al.*, 1994; Inoue *et al.*, 1995; Rawson and Hilbish, 1995). For example, diagnostic mtDNA markers were used by Rawson and Hilbish (1995) to describe the distributions of *M. galloprovincialis* and *M. trossulus* from San Diego to Seattle. Identification of species with mtDNA is, however, more difficult in mussels than in other groups of species, for salt (Mytilid) and freshwater (Unionid) species have both maternally and paternally inherited mtDNA systems (Skibinski *et al.*, 1994; Zouros *et al.*, 1992; 1994; Geller, 1996; Hoeh *et al.*, 1996; Liu *et al.*, 1996). The difficulties of using mtDNA as a diagnostic marker have been overcome by the use of a nuclear, codominant diagnostic marker. Inoue *et al.* (1995) described a diagnostic length difference in a gene that codes an adhesive protein found in byssal threads. The fragment may be amplified readily from genomic DNA, and the three species have distinctly different fragment sizes, making it simple to identify all three species and their hybrids, in which two fragments are coamplified.

The primary objectives of this study are to describe the macrogeographic and microhabitat distribution of three species (*M. edulis*, *M. trossulus*, and *M. galloprovincialis*) and the occurrence of their hybrids. Our sampling is more extensive than in previous studies, with multiple sites at some localities and a greater geographic extent of sample

sites. Finally, we have used a codominant, nuclear marker to identify the three species and their hybrids, and employed a second diagnostic marker to test for the presence of backcross genotypes within a hybrid zone.

Materials and Methods

Sample sites

Collection sites are shown in Figure 1 and described in Table 1. Mussels from Tillamook Bay, Coos Bay (1991), San Francisco Bay, Elkhorn Slough, Morro Bay, and San Diego Bay were the same as those reported upon in Geller *et al.* (1994). Additional mussels were obtained from Whidbey Island in the Puget Sound and shipped live to Wilmington, North Carolina, whereas mussels from Yokohama, Japan, were preserved in ethanol. DNA extractions and amplifications of these samples were conducted by JBG. The remaining samples were handled by BRK and JBM. Samples were shipped, either alive or frozen, to Boulder, Colorado, and held in an ultracold freezer (-80°C) until DNA was extracted.

DNA markers

DNA was extracted from the mantle tissue of each mussel by following the protocol of Geller *et al.* (1994).

In the study by Inoue *et al.* (1995) Me-15 and Me-16 PCR primers for a portion of an adhesive protein gene produced species-specific length variants of 180, 168, and 126 bp for *Mytilus edulis*, *M. trossulus*, and *M. galloprovincialis* respectively. Amplifications by BRK and JBM were conducted in a total volume of 25 μl using 10 mM Tris (pH = 8.3), 50 mM KCl, 0.01% gelatin, 200 μM dNTPs, 4 mM MgCl_2 , 1.5 units Taq DNA polymerase, 2 pmol of each primer, 0.75 μl DNA template, and autoclaved ultrapure water. Cycling conditions consisted of an initial denaturing step of 94°C for 45 s. This was followed by 35 cycles of 94°C for 45 s, 56°C for 30 s, 70°C for 90 s and concluded with a final extension step of 70°C for 3 min. Amplifications conducted by JBG used 10 mM Tris (pH = 8.3), 1.5 mM MgCl_2 , 50 mM KCl, 0.01% Triton-X 100, 0.01% gelatin, 0.01% NP-40, 200 μM dNTP's, 1.5 mM MgCl_2 , 0.3 units Taq DNA polymerase, 25 pmol of each primer, 0.5 μl DNA template, and autoclaved ultrapure water in a volume of 25 μl . Cycling parameters were 30 s at 98°C followed by 30 cycles of 10 s at 95°C , 30 s at 54°C , and 30 s at 72°C . For all amplifications, 10 μl of the PCR product were run on 3% agarose gels, stained with ethidium bromide, and visualized under UV light.

A pair of primers for a single copy anonymous nuclear DNA locus (Cmg-93) in a deep-sea hydrothermal vent vesicomyid clam, *Calypotegena magnifica* (Karl *et al.*, 1996), was found to amplify a single band of about 750 bp in *M. trossulus* but produced no product in either *M.*

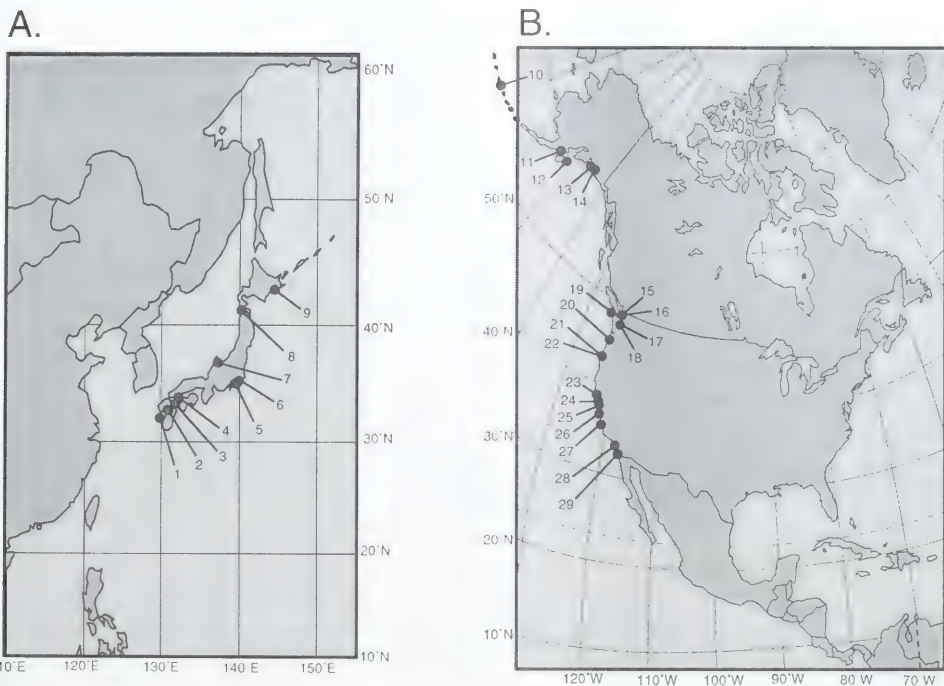


Figure 1. *Mytilus* collection sites in Japan (A) and North America (B).

edulis or *M. galloprovincialis*. This marker, in conjunction with the diagnostic marker of Inoue *et al.* (1995), permitted a more thorough analysis of genotypes in a zone of hybridization between *M. trossulus* and *M. galloprovincialis* at Palo Alto, California. F_1 hybrids between *M. galloprovincialis* and *M. trossulus* would have two bands at the Inoue marker, and a product for the Cmg-93 locus. An individual with two bands at the Inoue marker and no Cmg-93 product is one of several genotypes produced by backcrossing an F_1 hybrid with a specimen of *M. galloprovincialis*. The presence of this genotype clearly indicates that hybridization has extended beyond the F_1 level.

Amplifications of Cmg-93 were conducted by BRK and JBM in a total volume of 25 μ l using 10 mM Tris (pH = 8.3), 0.01% gelatin, 50 mM KCl, 300 μ M dNTPs, 2.5 mM $MgCl_2$, 1.5 units Taq polymerase, 6.25 pmol each of primer, bovine serum albumin (0.1 μ g/ μ l), 0.75 μ l DNA template, and autoclaved ultrapure water. Cycling conditions consisted of an initial denaturing step of 94°C for 60 s. This was followed by 35 cycles of 94°C for 60 s, 50°C for 60 s, 70°C for 60 s and concluded with a 70°C 2-min extension step. Ten microliters of the PCR product

were run on 1% agarose gels, stained with ethidium bromide, and visualized under UV light.

Annual and monthly (January and July) means for temperature and salinity for each site were taken from the NOAA World Ocean Atlas (1994). Data were obtained from the nearest point for each site from a $1^\circ \times 1^\circ$ grid of the North Pacific Ocean. In addition, some indication of the degree of wave exposure was recorded for most of the collection sites.

Results

On both sides of the North Pacific Ocean, *M. galloprovincialis* predominates in the lower latitudes, and *M. trossulus* predominates in the higher latitudes (Table 1, Fig. 1). In Japan, we found *M. galloprovincialis* alone at all sites on the southernmost island, Kyushu, and on Honshu, whereas only *M. trossulus* was found on the northern island of Hokkaido. *M. galloprovincialis* is most abundant in southern California, but only *M. trossulus* is found in Alaska. We did not detect *M. edulis* in any of our population samples.

Hybridization was not detected in the samples from

Table 1
The identity of individual Mytilus galloprovincialis, M. trossulus, and their hybrids in the North Pacific

Fig. # Ref. #	Location	Habitat type	Collected	Latitude/ longitude	Mean water temp (°C) annual (Jan/July)	Mean salinity (ppt) annual (Jan/July)	<i>Mytilus</i> <i>galloprovincialis</i>	<i>Mytilus</i> <i>trossulus</i>	Hybrids	Backcrosses
JAPAN										
Kyushu										
1	Amakusa	protected	1992	32°26'N; 130°06'E	22.36 (19.13/26.88)	34.24 (34.79/33.27)	6			
2	Kumamoto	protected	1996	32°30'N; 130°40'E	22.36 (19.13/26.88)	34.24 (34.79/33.27)	5			
Honshu										
3	Iwakuni	protected	1988	34°09'N; 132°11'E	22.90 (19.85/27.08)	34.49 (34.80/33.97)	28 [±]			
4	Hiroshima	protected	1996	34°24'N; 132°27'E	22.90 (19.85/27.08)	34.49 (34.80/33.97)	3			
5	Yokohama	protected	1993	35°28'N; 139°42'E	20.78 (16.68/24.68)	33.98 (34.29/33.60)	3 [±]			
6	Chiba (Kominato)	protected	1992	35°36'N; 140°07'E	18.64 (14.17/23.17)	33.10 (33.51/32.61)	6			
7	Toyama	protected	1988	36°42'N; 137°14'E	17.69 (11.70/22.75)	33.76 (33.82/33.74)	7 [±]			
8	Mutsu Bay (Aomori)	semi-exposed	1992	41°05'N; 140°55'E	15.14 (7.73/20.31)	33.81 (33.75/33.75)	6			
9	Hokkaido Akeshi Bay	protected	1992	43°18'N; 145°31'E	11.72 (2.31/13.39)	32.98 (32.96/32.93)		10		
ALASKA										
Alutian Isl.										
10	Adak Isl.	very protected	1992	51°50'N; 176°40'W	6.80 (3.54/7.73)	32.93 (32.99/32.90)		2		
10	Adak Isl.	protected	1992	51°50'N; 176°40'W	6.80 (3.54/7.73)	32.93 (32.99/32.90)		4		
10	Adak Isl.	very exposed	1992	51°50'N; 176°40'W	6.80 (3.54/7.73)	32.93 (32.99/32.90)		2		
Gulf of Alaska										
11	Kinak Bay	exposed	1992	58°06'N; 152°53'W	7.79 (4.40/10.26)	31.70 (32.27/31.62)		13		
12	Ouzinki Pass	protected	1992	57°55'N; 152°30'W	7.87 (4.53/10.38)	31.84 (32.26/31.71)		12		
Prince William Sound										
13	Knight Isl. (Herring Pt.)		1992	60°29'N; 147°46'W	8.40 (5.31/11.35)	31.38 (31.93/31.01)		2		
13	Knight Isl. (Herring Bay)		1992	60°27'N; 147°42'W	8.40 (5.31/11.35)	31.38 (31.93/31.01)		2		
14	Chenequa Isl.		1992	60°23'N; 148°00'W	8.40 (5.31/11.35)	31.38 (31.93/31.01)		2		

Table 1 (Continued)

Fig. 1 Ref. #	Location	Habitat type	Collected	Latitude/ longitude	Mean water temp (°C) annual (Jan/July)	Mean salinity (ppt) annual (Jan/July)	<i>Mytilus</i> <i>galloprovincialis</i>	<i>Mytilus</i> <i>rossius</i>	Hybrids	Backcrosses*
WASHINGTON										
San Juan Isl.										
15	Eagle Cove	semi-exposed	1992	48°28'N; 123°02'W	12.06 (8.96/14.35)	31.01 (31.02/30.86)		6		
16	Argyle Creek	protected	1992	48°32'N; 123°01'W	12.06 (8.96/14.35)	31.01 (31.02/30.86)		6		
Puget Sound										
17	Whidbey Isl. (Penn Cove)	protected	1994	48°13'N; 122°41'W	12.06 (8.96/14.35)	31.01 (31.02/30.86)	5	13	2	
18	Skookum Bay	protected	1992	47°09'N; 123°03'W	12.46 (9.38/14.81)	30.99 (31.06/30.77)		7		
19	Tatoosh Isl.	exposed	1996	48°23'N; 124°44'W	12.04 (8.84/14.33)	31.39 (31.47/31.22)		5	1	
OREGON										
20	Tillamook Bay (Marshall Coos Bay)	protected	1991	45°23'N; 128°59'W	13.06 (9.96/14.87)	31.60 (31.78/31.28)		17		
21	Isthmus Slough	protected	1991	43°22'N; 124°12'W	13.04 (10.05/13.74)	32.43 (32.51/32.30)		25		
21	Isthmus Slough	protected	1996	43°22'N; 124°12'W	13.04 (10.05/13.74)	32.43 (32.51/32.30)	4	2		
22	South Slough	protected	1992	43°20'N; 124°19'W	13.04 (10.05/13.74)	32.43 (32.51/32.30)		12		
CALIFORNIA										
Tonales Bay										
23		protected	1992	38°11'N; 122°54'W	13.81 (11.99/13.65)	32.92 (32.90/33.50)	9			
24	San Francisco Bay (North Beach)	protected	1992	37°49'N; 122°24'W	13.48 (11.71/13.05)	32.95 (32.96/33.12)	16	26	5	
25	Palo Alto (Yacht Club)	protected	1990	37°29'N; 122°06'W	13.98 (12.42/13.91)	33.06 (33.06/33.17)	10		2	2*
25	Palo Alto (Yacht Club)	protected	1996	37°29'N; 122°06'W	13.98 (12.42/13.91)	33.06 (33.06/33.17)	25		10	15*
26	Elkhorn Slough	protected	1992	36°35'N; 121°55'W	13.98 (12.42/13.91)	33.06 (33.06/33.17)	21		2	
27	Morro Bay	exposed	1992	35°48'N; 120°56'W	14.14 (12.71/14.39)	33.25 (33.25/33.34)	25		1	
San Diego										
28	Scripps Pier	exposed		32°51'N; 117°17'W	16.02 (15.02/17.69)	33.52 (33.48/33.58)	10			
29	San Diego Bay	protected	1992	32°45'N; 117°08'W	16.02 (15.02/17.69)	33.52 (33.50/33.54)	18	3	11	

* The use of two sets of primers in the Palo Alto samples allowed discrimination of F1 hybrids and backcrosses. See text for further details.

† These individuals were cultured from ballast water from ships originating from these ports.

‡ Collected as drift from a protected beach.

Japan, but hybrids were common in San Diego Bay and San Francisco Bay. Both *M. galloprovincialis* and *M. trossulus* and their hybrids were detected at Whidbey Island, near a mussel farm that raises *M. galloprovincialis*. A single hybrid was found in a sample from Tatoosh Island, Washington.

The patchy distribution of species is apparent in and around the bays of California. For instance, only *M. galloprovincialis* was present on the outer coast at Scripps Pier in San Diego, but just a few kilometers away, *M. galloprovincialis*, *M. trossulus* and their hybrids were found in San Diego Bay. In San Francisco Bay, near the Golden Gate Bridge at North Beach, both species and hybrids were common (34% *M. galloprovincialis*, 11% *M. trossulus*, 55% hybrids). However, deeper in the bay, at Palo Alto, *M. trossulus* was apparently absent, but *M. galloprovincialis* and hybrids were common. The individuals collected from Palo Alto were analyzed with the two diagnostic markers to examine the extent of hybridization (Table I). In both 1990 and 1996, we identified genotypes consistent with F_1 hybrids (14% and 20%, respectively) and backcrosses (14% and 30%, respectively) of F_1 hybrids to *M. galloprovincialis*.

In Isthmus Slough and South Slough within Coos Bay, only *M. trossulus* was found in 1991. However, a sample taken from Isthmus Slough in 1996 contained both *M. galloprovincialis* and *M. trossulus*. It is not known whether the difference between the two samples taken from Isthmus Slough reveals a change over time or pre-existing microgeographic variation.

Although our survey identified microgeographic variation in species distributions, an environmental cause was not apparent. For our collection sites, neither wave exposure (Suchanek, 1978) nor salinity supplied any predictive power to explain the distribution of species.

Despite the lack of any explanative value in environmental conditions at a microgeographic scale, on a broader geographic scale the transitions between *M. galloprovincialis* and *M. trossulus* occur between 40° and 41° north latitude on both sides of the Pacific. Data in Table I allow a comparison of temperature regimes across the transition zones. At the break on the eastern side of the Pacific, there are only slight differences in mean temperatures (compare sites 22 and 23). The only appreciable difference is a drop of about 2°C in the mean January temperature at the northern site (site 22). However, a comparison of sites 8 and 9 in Japan reveals substantial differences in temperature across the transition zone. The northern site not only has a lower mean January temperature (by about 5.5°C), but also lower mean annual and mean July temperatures (by 3°C and 7°C, respectively). A comparison of the eastern and western sites at the southern boundary of pure *M. trossulus* (sites 9 and 22) reveals that mean January temperatures are quite different (2.21° and 10.5°C, respectively), but that annual

mean temperatures (11.72° and 13.74°C, respectively) and mean July temperatures (13.39° and 13.74°C, respectively) are similar.

Discussion

In comparison to previous studies, our finer scale geographic sampling of *Mytilus* in California revealed a more patchy distribution of pure species and a greater geographical extent of hybrids. Whereas previous studies had suggested that pure *M. galloprovincialis* extended as far north as Point Conception, we found a population of pure *M. galloprovincialis* in Tomales Bay, 700 km further north. Furthermore, our studies of populations on both sides of the Pacific revealed that the transition zones are at similar latitudes, and at similar temperatures, suggesting that temperature influences the distributions of these species.

Hybridization and patchiness

The authors of a previous study (Rawson and Hilbish, 1995) concluded that hybridization of *M. galloprovincialis* and *M. trossulus* was rare in southern California. They sampled from the pier at the Scripps Institution of Oceanography, and they found only *M. galloprovincialis*. Similarly, our sample from the same site also yielded only *M. galloprovincialis* (Table I), but more extensive sampling inside San Diego Bay in a small-craft marina revealed both species and their hybrids.

Although earlier reports indicated that hybridization was evident in San Francisco Bay (McDonald and Koehn, 1988; Sarver and Foltz, 1993) and relatively infrequent elsewhere (Rawson and Hilbish, 1995), this survey revealed hybrids in San Diego Bay, Elkhorn Slough, two areas within San Francisco Bay, and two sites in Washington. San Francisco Bay and Elkhorn Slough are located near the southern boundary for continuous populations of *M. trossulus* and the northern boundary for continuous populations of *M. galloprovincialis*, so hybridization there is not surprising. Studies of mussels in San Francisco Harbor (Rawson *et al.*, 1996) and the present study (Table I) revealed that the proportions of pure species, F_1 hybrids, and backcrosses vary across the bay, with *M. galloprovincialis* becoming more common deeper in the bay. The studies of mussels from Palo Alto clearly indicate that hybridization and backcrossing are common, and that the mixing appears to be persistent over time. Presence of hybrids away from this contact zone around San Francisco requires further explanation. The San Diego Bay site is in a marina where small craft may have introduced adult or larval *M. trossulus*, initiating hybridization. *M. galloprovincialis* and hybrids at Whidbey Island may be progeny of cultured mussels in a nearby mariculture facility. A hybrid at Tatoosh Island is possibly an indication of transport of hybrid larvae from Puget Sound.

Sporadic episodes of transplantation by means of ships will cause the presence of *Mytilus* species and their relative abundances to fluctuate over time. Although the adults are effectively sedentary, the pelagic larvae are in the water column for 3 weeks or more; depending on the currents, they may move distances in excess of 200 km. But in addition to their natural dispersal, mussels are moved about extensively on the hulls of and in the ballast water of ships. Freighters moving between continents can inoculate a bay with many millions of larvae, and thus the distributions and the relative numbers of species of *Mytilus* might change from year to year (Carlton and Geller, 1993).

The absence of *M. edulis* in our samples, as in previous allozyme and mtDNA reports, remains enigmatic, for there are several reports that *M. edulis* has been transplanted to the west coast of North America. For example, hundreds of oyster epibionts, which include *M. edulis*, have been accidentally released in Pacific sites (Carlton, 1989). Furthermore, we suspect that individuals of *M. edulis* are introduced regularly to western ports by cargo ships discharging ballast water (Carlton, 1989; Carlton and Geller, 1993). *M. edulis* has subsequently been identified (D. Heath, pers. comm.) from a sample of alien mussels (Heath *et al.*, 1995) collected in the Georges Strait, north and west of Vancouver, near an area where *M. edulis* is farmed.

Transition zones

In California, the transition between *M. galloprovincialis* and *M. trossulus* is near 40° or 41°N latitude, approximately the Cape Mendocino region. Previous studies had suggested that the northern limit of pure *M. galloprovincialis* was at Point Conception or Morrow Bay (Sarver and Loudenslager, 1991; Rawson and Hilbish, 1995), but our study revealed pure *M. galloprovincialis* in Tomales Bay, nearly 700 km further north. This transition is far north of the biogeographic boundary at Point Conception, a recognized demarcation between cold-water and warm-water biotas (Valentine, 1966), where many molluscan species have a distributional limit.

Which variables control the latitudinal distributions of *M. galloprovincialis* and *M. trossulus*? Temperature is generally believed to control distributional boundaries of marine organisms (Valentine, 1966), and *Mytilus* is no exception (Seed, 1976). *M. edulis* is able to withstand freezing in ground ice at -20°C for 6 to 8 months of the year (Williams, 1970), but the northern distribution for *M. edulis* may be limited by the temperatures that permit breeding (e.g., Stubbings, 1954; Barnes, 1957). Similarly, the southern limit of the range of *M. edulis* in the western North Atlantic is determined by summer temperatures, which are lethal for *M. edulis* on the Outer Banks of North Carolina (Wells and Gray, 1960).

In both the eastern and western Pacific, the transition zones between *M. galloprovincialis* and *M. trossulus* occur where the annual mean temperature is between 13° and 14°C. On both sides of the Pacific, the southernmost populations of pure *M. trossulus* (sites 9 and 22) experience mean July temperatures of 13.39° and 13.74°C, respectively. We hypothesize that summer temperatures warmer than 13°–14°C approach the upper thermal limit for *M. trossulus* and reduce the viabilities of larvae and adults. Although historically *M. trossulus* was established south of this latitude in California, it was probably restricted to cooler sites. Geller (unpubl. data) used mitochondrial 16S rRNA gene sequences to identify *M. trossulus* collected from Catalina Island in 1900 and from Monterey Bay in the 1870s. Both Catalina Island and Monterey Bay are cooler than the collection sites employed in the present study in San Diego Bay. We do not propose that the temperature of 14°C is an absolute barrier, for we sampled *M. trossulus* from San Diego Bay (mean annual temperature = 16.02°C, mean July temperature = 17.69°C). However, the presence of *M. trossulus* within San Diego Bay but not on the open coast, at the Scripps Pier, suggests that persistent reintroduction by means of intracoastal boating is necessary to maintain *M. trossulus* in the relatively warm localities in southern California. If our hypothesis is correct, *M. trossulus* would not persist in San Diego Bay without recurrent introductions by ships.

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Reports of Papers Presented at the General Scientific Meetings of the Marine Biological Laboratory August 18 to 20, 1997

Program Chairs:

Robert Paul Malchow, University of Illinois at Chicago
Mark Martindale, University of Chicago
Charles Hopkinson, Ecosystems Center, MBL

Each of these reports was reviewed by two members of a special editorial board. This board was drawn from the research community of Woods Hole, Massachusetts, and included scientists from the Marine Biological Laboratory, the Woods Hole Oceanographic Institution, and the National Marine Fisheries Service.

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ABSTRACTS

In addition to the work described here in Short Reports, the following papers were also presented. The abstracts of these papers are available from the Marine Biological Laboratory Archives, 7 MBL Street, Woods Hole, MA 02543.

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- Responses of goldfish Mauthner cell to auditory stimuli

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Roberts, Brian J., Jane Tucker, and Anne Giblin

Effect of MnO_2 on denitrification in marine sediments

Wicklein, Martina, and John Layne

Looming-sensitive interneurons in the optic ganglia of the fiddler crab *Uca minax* (Crustacea: Decapoda)

Introduction to Featured Article: The Lateral Eyes of Two Species of Horseshoe Crabs Are Similar, but Not Quite the Same

The lateral eye of the horseshoe crab has been a useful and productive model for vision research; it is complex enough to be interesting, yet simple enough to be understood. Extensive studies of this eye have yielded fundamental insights about how eyes of other animals, including humans, encode and process visual information. Lateral inhibition, light adaptation, and efferent-mediated circadian rhythms are just a few of the numerous physiological properties that were first uncovered through experiments on the horseshoe crab eye.

We always speak about “THE horseshoe crab,” and indeed, all of the studies and advances have been made with the eye of a particular species, *Limulus polyphemus*, which inhabits the waters along the eastern shores of North and Central America. But there are actually four extant species of horseshoe crabs—one of them, *Tachypleus tridentatus*, inhabits the shores of Japan. In the following article, T. Saito and his colleagues compare the properties of the eyes of *Tachypleus* with those of the standard model, *Limulus*.

Saito *et al.* have found that, although the retinal sensitivity of the lateral eyes of *Tachypleus* and *Limulus* shows nearly the same circadian rhythms, the underlying mechanisms are different. The *Limulus* eye increases in sensitivity at night primarily by catching more photons, whereas the *Tachypleus* eye shows, in addition, a substantial increase in photoreceptor gain; *i.e.*, an enhanced response of the photoreceptors to absorbed photons.

This difference could be explained by the fact that the lateral eyes of *Tachypleus* are smaller than those of *Limulus* (see Fig. 1); moreover these smaller eyes contain fewer, smaller ommatidia that cannot catch as many photons as those of *Limulus*. So the increased response of the *Tachypleus* photoreceptors to each photon they absorb may be a compensatory adaptation. In short, what the small *Tachypleus* eye lacks in photon catch, it may make up in gain.

Once again we find that although the functions of homologous tissues and cells are similar, they are never exactly the same, and that important and unexpected lessons can be learned in even small species differences.

—Robert B. Barlow
August 1997

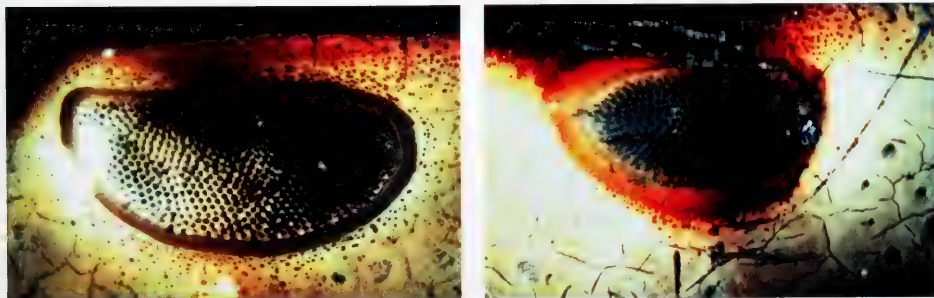


Figure 1. The lateral eyes of *Tachypleus* (right) and *Limulus* (left). The photographs are at the same magnification; the *Limulus* eye is 1 cm wide.

Circadian Rhythms in the Lateral Eye of the Japanese Horseshoe Crab

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(Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

The visual system of the "American" horseshoe crab, *Limulus polyphemus*, provides a clear example of the brain modulating a sensory input. At night, efferent optic nerve fibers transmit neural signals to the lateral eyes from a circadian clock located in the brain (1). The efferent signals change the structure and function of the lateral eye (2), increasing its sensitivity to such a level that the animals can find mates underwater at night (3,4). These circadian rhythms in retinal sensitivity persist in constant darkness and can be phase shifted by exposure to light pulses at various times during the circadian cycle (5). Although four extant species of horseshoe crabs inhabit various oceans of the world (6), circadian rhythms have been detected only in *Limulus polyphemus*, which inhabits the eastern shores of North and Central America. Here we report that circadian rhythms occur in the visual system of the Japanese horseshoe crab, *Tachypleus tridentatus*, and differ in at least one property from those in *Limulus*.

Three adult *Tachypleus* males were shipped to the Marine Biological Laboratory (Woods Hole, Massachusetts) from the Horseshoe Crab Museum (Kasaoka, Okayama, Japan). We were fortunate to receive these animals because they are protected by the Japanese government as a result of the recent precipitous decline in their population. Before experimentation, both *Tachypleus* and *Limulus* were entrained to natural environmental lighting for at least five weeks in aquaria at the Marine Biological Laboratory. Following techniques described elsewhere (5), we mounted a crab on a rigid platform in an aquarium contained in a lightproof, shielded cage and, using corneal electrodes, recorded electroretinograms (ERGs) from their dark-adapted lateral eyes in response to 20-ms light flashes generated by a green LED. We measured the peak-to-peak response of the ERG, which provides a convenient measure of the summed response of photoreceptor cells to brief flashes of light (5). We measured circadian rhythms in the photoreceptor response by recording ERGs every 15 min over several days as the animal remained in constant darkness. We assessed changes in photoreceptor sensitivity by measuring the intensity-response function of the ERG response. To avoid noncircadian changes in sensitivity (5), we recorded the intensity-response functions and other circadian properties after the animals were kept in darkness >24 h.

The *Tachypleus* lateral eye exhibits a circadian rhythm as does that of *Limulus*. The ERGs recorded from both species increase in amplitude at about the time of dusk, remain high throughout the subjective night, decrease near dawn, and remain

low during the subjective day. As reported elsewhere (5), the period of the endogenous rhythm in *Limulus* eye ERG ranges in duration from 22.2 to 25.5 h with a mean value of 23.9 ± 0.7 h ($n = 75$). Using the same measurement method (5), the periods of the circadian oscillations recorded from the eyes of three *Tachypleus* were 24.3 h, 24.3 h, and 23.5 h in duration. That of the *Limulus* eye was 24.1 h. As reported elsewhere, the phase of the circadian rhythm in *Limulus* can be advanced up to 3 h and delayed up to 4 h by exposing the animal to brief periods of illumination at various times during the circadian cycle (5). We found that exposing *Tachypleus* to 1 h of light at CT 15 (see legend for definition of CT) delayed the phase of the ERG rhythm by 4 h. In sum, the endogenous rhythm in the ERG of the *Tachypleus* eye free runs with a period of about 24 h and can be phase shifted. We conclude that, as in *Limulus*, a circadian clock is a fundamental component of the *Tachypleus* visual system.

How do changes in ERG amplitude relate to changes in photoreceptor sensitivity? Figure 1 plots the amplitudes of ERGs recorded from dark-adapted *Tachypleus* and *Limulus* eyes on log scales as a function of log light intensity. The "Day" data (open circles) for *Limulus* were fitted by eye with a smooth curve. This same curve shifted 0.9 log units to the left approximately overlays the "Night" data (filled circles), indicating that

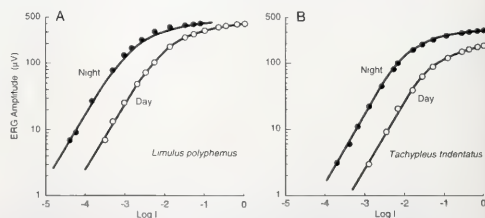


Figure 1. Intensity-response functions for ERGs recorded from dark-adapted lateral eyes of *Tachypleus* and *Limulus*. The ERG amplitude is plotted on a log scale on the ordinate as a function of log light intensity plotted on the abscissa. The "Day" data were recorded near the middle of the second subjective day (CT 8 for *Tachypleus* and CT 7 for *Limulus*), and the "Night" data were recorded near the middle of the second subjective night (CT 17 *Tachypleus* and CT 15 for *Limulus*). Retinal sensitivity had reached steady levels at each of these circadian times. The circadian time of 0 h (CT0) is defined as the animal's subjective dawn and CT 18 is the middle of its subjective night. Refer to (5) for the technique for measuring circadian times for ERG rhythms. Repeated measurements 24 h later yielded nearly identical functions, with ERGs greater than 50 μ V varying <5% and those less than 50 μ V varying <30%. The maximum intensity (Log I = 0) incident on the surface of the cornea was 19.5 μ W/cm² at $\lambda = 520$ nm (Model 262 photodiode, Graseby Corp., Orlando, FL).

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the increased sensitivity of the nighttime state caused a lateral shift of the intensity-response function. Such a shift is consistent with the idea that clock-induced structural changes in the ommatidia at night allow photoreceptor cells to absorb more photons than during the day (2). The shift of 0.9 log units corresponds to ~8-fold increase in photons absorbed at night and is consistent with previously reported results (5). We conclude that responses of *Limulus* photoreceptors to brief flashes increase at night, not because they are intrinsically more sensitive to light, but because they absorb more photons.

The intensity-response functions for the *Tachypleus* lateral eye appear similar to those for *Limulus*, but there are important differences. The "Day" and "Night" functions are similar in the sense that they can be fitted by the same curve used for the *Limulus* data in Figure 1A, indicating that increases in photoreceptor responses to increases in light intensity are about the same in both species. At low flash intensities, the ERG amplitudes for both species are linearly related to light intensity over ~2 log unit range; that is, a 10-fold increase in intensity produces about a 10-fold increase in response amplitude. One clear difference between the *Tachypleus* and *Limulus* intensity-response functions is that those of *Limulus* are shifted to the left on the intensity axis; that is, the *Limulus* eye generates significantly larger ERGs in response to the same stimulus intensities.

Another significant difference between *Tachypleus* and *Limulus* intensity-response functions is that the "Day" curve for *Tachypleus*, unlike that of *Limulus*, cannot be shifted horizontally to match the "Night" curve. However, shifting the "Day" curve 0.6 log units to the left and then 0.23 log units vertically provides a match to the "Night" curve. The 0.6 log unit horizontal shift corresponds to only a 4-fold increase in photons absorbed by photoreceptors at night; this is half that of *Limulus* photoreceptors. However, the 0.23 log unit vertical shift corresponds to a 1.7-fold increase in gain of *Tachypleus* photoreceptors. *Limulus* photoreceptors show no comparable increase in gain in their responses to brief flashes. A previous study shows that *Limulus* photoreceptors attain an increased gain at night only for steady-state responses (7).

Examination of the lateral eyes of adult *Tachypleus* males shows that they contain fewer and smaller ommatidia than the *Limulus* eye. They are also smaller than *Limulus* eyes: the *Tachypleus* and *Limulus* eyes recorded in Figure 1 contained 452 and 733 ommatidia, respectively; the ommatidia were 143 and

193 μm in diameter; and the eyes had retinal areas of 16.6 and 27.8 mm^2 .

Both *Tachypleus* and *Limulus* exhibit circadian rhythms in lateral eye sensitivity at night. Although the nighttime increase in sensitivity is about the same for both species, the underlying circadian mechanisms are not. Both *Tachypleus* and *Limulus* photoreceptors catch more photons at night, but *Limulus* photoreceptors are twice as effective, exhibiting an 8-fold increase in photon catch to only a 4-fold increase for *Tachypleus*. Circadian changes in retinal structure mediate the nighttime increases in photon catch in *Limulus* (9). Because photon catch is proportional to the cross-sectional area of ommatidial corneal facets, a single *Limulus* ommatidium ($0.029 \text{ m}\mu^2$) should catch ~1.8 times more photons than a *Tachypleus* ommatidium ($0.016 \text{ m}\mu^2$). *Tachypleus* photoreceptors, on the other hand, acquire greater gain at night, generating responses to brief flashes that are about 1.7 greater in amplitude. The nighttime increase in gain of *Tachypleus* photoreceptors appears to compensate for their smaller increase in photon catch.

Higher visual sensitivity at night helps *Limulus* males find mates (3). No behavioral studies have been carried out thus far with *Tachypleus*.

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Prediction of Maximum Allowable Retinal Slip Speed in the Fiddler Crab, *Uca pugnator*

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Stabilizing eye movements reduce movement of the image on the retina (retinal slip), and thus are assumed to improve visual acuity by reducing blur. The reasoning behind this argument is as follows. The response of individual photoreceptors (in the form of depolarization) to a brief flash of a given intensity is also brief. As is commonly observed in synaptic summation, the response to longer flashes is essentially a summation of many short-flash responses. After a period approximately equal to the duration of one short-flash response (T_s), the summation ceases, and the response reaches a steady state. Prolonging the flash does not further depolarize the cell. If a photoreceptor then samples a particular direction in space for more than T_s , it gains no new information. Assuming no change in the intensity of incident light, this means that the eye does not lose spatial information if a retinal slip equal to one visual angle per T_s (1,2) occurs. Here we present two measurements of the temporal dynamics of photoreceptors in the fiddler crab, *Uca pugnator*: T_s and the frequency response. We will consider these in light of the theoretical allowable slip speeds for this animal.

ERG measurements. The flash response time of *Uca* photoreceptors was estimated from ERG measurements after the crabs had been adapted for four hours to total darkness in the recording chamber. Light flashes ($\lambda = 570$ nm, $1.25 \mu\text{W}/\text{cm}^2$) of increasing length were delivered at a frequency of 1 Hz, and the average amplitude of 15–20 ERGs was recorded. The flash duration at which the maximum ERG amplitude was reached and maintained for three consecutive measurements was taken as the length of the flash response for a dark-adapted eye. The flicker fusion frequency was measured with 10 ms flashes in dark adapted crabs, as well as in crabs adapted to and tested in $11.65 \mu\text{W}/\text{cm}^2$ fluorescent light. LED flashes of 570 nm, 10 ms long, were $1.25 \mu\text{W}/\text{cm}^2$ higher than adaptation and background intensity.

Photoreceptor acceptance angle. This value, ζ , was estimated from the inter-ommatidial angle, $\Delta\phi_b$, which gives the spatial sampling frequency of the receptor array. The interommatidial angle was derived from pseudopupil mapping (3). While ζ for a given photoreceptor is not necessarily equivalent to $\Delta\phi_b$, it can be assumed for theoretical reasons to be close to but greater than $\Delta\phi_b$ (4).

The values of T_s and ζ allow a conservative estimate of the highest retinal slip speed that does not decrease spatial acuity. This estimate is conservative because we have underestimated the ζ by using $\Delta\phi_b$, and we have overestimated T_s by using

dark adapted animals, which have a longer flash response than light adapted ones (5). Because $\text{slip speed}_{\text{max}} = \zeta / T_s$, any variation from the values used here will increase the allowable slip speed.

Retinal slip speed. The retinal slip velocity tolerated by a crab was measured as follows: thin wires were attached to the eyestalks projected horizontally across the back of the crab. Crabs were allowed to walk freely on a platform within a striped drum turning at 6°/s and illuminated at the same intensity used for light adaptation. Behavior was videotaped at 60 fields/s. Retinal slip velocities were measured by digitizing the wire

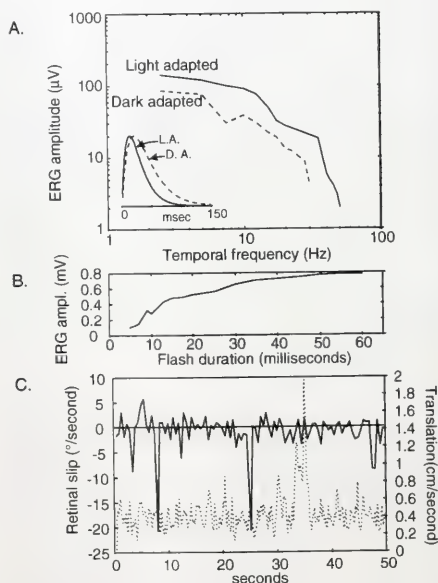


Figure 1. ERG amplitude in response to variation in frequency (A) and duration (B) of LED flash. A. The flicker fusion frequency for these conditions was approximately 32 Hz for dark-adapted (dashed line) and 50 Hz for light-adapted (solid line) animals, respectively. The impulse response functions for dark and light adapted crabs, estimated from the frequency response data, are shown in the inset. B. The saturation time duration of the response to a flash of light was approximately 31 ms. C. Retinal slip speeds (solid line) observed in a freely walking crab surrounded by a rotating striped drum (6°/s, left-hand axis). Crab translational velocity (dotted line) in cm/s during the same period (right-hand axis).

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between every other field, calculating the angular velocity of the eye, and subtracting the drum velocity.

Figure 1A shows the ERG amplitude in μV as a function of LED flash frequency (left-hand axis). The ERG amplitude modulation became vanishingly small at 32 Hz and 50 Hz, for dark- (dashed line) and light- (solid line) adapted animals, respectively. Figure 1B shows the ERG amplitude as a function of flash duration. The value at which the response saturates is about 65 ms in a dark-adapted crab. This estimate of T_i is corroborated by the frequency response data in Figure 1A. If we Fourier-invert the frequency data to get the impulse response, we find that this is well fitted by a 2nd order transfer function, which corresponds to an impulse function of the form $t(\exp(-t/\tau))$ (see Fig. 1A inset). From this we can estimate a time constant (τ) of about 14 and 21 ms in light- and dark-adapted crabs, respectively. The great majority of the energy in the impulse response occurs within 3τ , giving an estimate for dark adapted animals of 63 ms. This value, along with $\Delta\phi_h \cong \zeta = 2.1^\circ$, means that the crab should not lose spatial resolution with a retinal slip velocity $\leq 33.3^\circ/\text{s}$. Figure 1C shows a representative plot of retinal slip in a crab walking inside a drum (upper trace). The peaks in slip velocity represent saccadic eye movements, in which the animal moves its eyes to a new position as it turns. Clearly, the retinal slip that is tolerated rarely exceeds $3^\circ/\text{s}$, even during fast locomotory movements, indicated in the lower trace (right-hand axis).

The flicker fusion value is indicative of the retinal slip speed at which *all* spatial information is lost, whereas $33.3^\circ/\text{s}$ is the

velocity at which spatial information *begins* to be lost. This far exceeds observed retinal slip speeds, suggesting some other reason for image stability unrelated to spatial acuity. Indeed, this maximum slip speed limit is only approached in animals that actively scan the environment with their eyes, primarily to enlarge the visual field [spiders, planktonic mollusks and crustaceans (6), but see stomatopods (2)]. As for fiddler crabs, which do not scan, we can only speculate that the image on the retina must be extremely stable for successful motion vision, especially the detection of optic flow; image slip would pollute the optic flow pattern, and reduce the information that might be gained therefrom.

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Histamine: Putative Transmitter for Lateral Inhibition in *Limulus* Eye

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Lateral inhibition is an integrative mechanism found in many nervous systems. In retinas, it enhances borders and edges in the visual field (1). Since its discovery in the lateral eye of the horseshoe crab, *Limulus polyphemus*, by H. Keffer Hartline and colleagues in 1952 (2), lateral inhibition has been studied extensively (3). It is now the foundation of a comprehensive model of retinal function (4). One question, however, remains unanswered. What is the neurotransmitter that mediates lateral inhibition in the *Limulus* eye? Histamine has been implicated as a photoreceptor neurotransmitter in Unirama and Crustacea, two of the major groups of arthropods (5, 6, 7). In preparations that study the postsynaptic effects of histamine, histamine has been shown to be inhibitory (7, 8). Biochemical studies show that histamine and the enzymes that synthesize it are present in the *Limulus* retina (9), but provide no physiological evidence that histamine is the transmitter of lateral inhibition. In this paper, we report electrophysiological and pharmacological studies that test the role of histamine in lateral inhibition in the *Limulus* eye.

In the first experiment, we tested whether histamine mimics the action of lateral inhibition on optic nerve activity generated by eccentric cells in individual ommatidia. To gain access to eccentric cells, we excised the lateral eye and sectioned it into three or four slices. One slice was placed in a Lucite perfusion chamber (volume 0.5 ml). A glass microelectrode filled with 3 M KCl (25 M Ω) was connected to a DC bridge amplifier (Electronics Shop, Rockefeller University) and advanced into an ommatidium using a Burleigh Inchworm (Burleigh Instruments, Fishers, NY) attached to a manipulator. A fiber optic light pipe was aligned with the optical axis of the ommatidium for maximal stimulation. In this manner, we recorded intracellular responses of eccentric cells to 8-s light flashes delivered every 5 min. After a series of control runs in which a modified *Limulus* Ringer was perfused over the slice (rate: 1 ml/min), a 1 mM solution of histamine in saline was applied to the slice at the same rate.

Figure 1A shows responses of an eccentric cell before treatment, during the application of histamine, and after washout

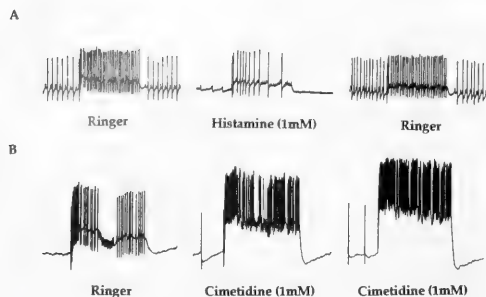


Figure 1. The action of histamine and cimetidine on eccentric cells of the *Limulus* lateral eye. (A) Eighty minutes of perfusion with 1 mM histamine reduced both the spontaneous and light driven response of the eccentric cell (57 mV resting potential). Washout with modified *Limulus* Ringer reversed the effect of histamine. The amplitude of the largest spontaneously evoked action potential was 45 mV, but appears in these traces as 18 mV because of the low pass characteristics of the Gould Brush recorder. Note that the intracellular recordings contain action potentials of different amplitudes. The smaller one is generated by a second eccentric cell in the same ommatidium and is detectable because of partial coupling among ommatidial cells. The existence of multiple eccentric cells in single ommatidia is not uncommon and can be as high as 20% (R. Barlow, unpub. obs.). (B) Twenty minutes of perfusion with 1 mM cimetidine reduced the action of lateral inhibition and forty minutes of perfusion abolished it. Forty-five minutes of Ringer washout partially reversed the cimetidine effect (data not shown). Artifacts caused by antidromic shock delivered to the optic nerve trunk are detectable in the middle of the first trace. The amplitude of the spontaneously evoked action potential in the second trace was 65 mV, but was attenuated to 24 mV by the pen recorder. All traces are 18 s in duration.

with modified *Limulus* Ringer. The cell fired large (45 mV) action potentials in response to dim background illumination (~ 1.8 impulses/s) before the light flash. A second eccentric cell located in the same ommatidium generated much smaller action potentials (~ 2.5 mV) with the same firing rate (see legend). While perfusing the slice with modified *Limulus* Ringer (first trace), the light flash evoked a burst of ~ 7 action potentials at the leading edge of the generator potential, followed by 34 action potentials yielding a steady-state firing rate of ~ 4.5 impulses/s for the last 4 s of the response. The second trace was recorded after 80 min of perfusion with histamine and shows its maximal effect on this cell. Notice that the cell's activity decreased both in response to the dim background and to the light flash. Only 2 spikes fired at flash onset, followed by 9 spikes for a steady rate of ~ 0.75 impulses/s. Thus the response rate of the cell was reduced by $> 80\%$, or 3.75 impulses/s. Note that the response of the second eccentric cell to background illumination declined by $> 50\%$, but we could not assess its response to the light flash because of masking by the larger action potential. Ringer washout reversed the action of histamine. The third trace shows that after a 55 minute washout, the light flash evoked 33 action potentials with a steady state rate of ~ 4.0 impulses/s. The response rate of the cell thus returned to $\sim 90\%$ of its original level. Reapplication of histamine de-

creased the steady state response rate of the cell again to ~ 0.75 impulses/s after 180 min of perfusion (data not shown). As before, the number of action potentials rose after washing out the histamine. In general, the initial effects of histamine were detected about 20 min after application, while maximal effects occurred about 50 min later. In all experiments in which histamine was tested ($n = 3$), it reduced the optic nerve activity generated by eccentric cells.

In a second type of experiment, we tested whether cimetidine, a blocker of histaminergic receptors, could reduce the effects of lateral inhibition on eccentric cell responses. To do this, we excised the lateral eye from the animal in such a way as to retain ~ 0.5 cm of the optic nerve attached to the back of the eye. We gained access to ommatidia by slicing away the ventral half of the eye without cutting the optic nerve trunk. The eye was then mounted in a chamber, and the optic nerve was pulled into a suction electrode. Following the procedures described above, we impaled single eccentric cells and recorded their responses to light with and without lateral inhibition exerted by antidromic stimulation of the optic nerve trunk. Current pulses applied through the suction electrode exerted massive lateral inhibitory effects in the cell impaled by our electrode. After control runs in which the eye was perfused with modified *Limulus* Ringer, 1 mM cimetidine was applied to the preparation.

Figure 1B shows the response of an eccentric cell to both light and lateral inhibition before and during the application of cimetidine. The light flash was 10 seconds in duration. Four seconds after light onset, optic nerve shock was delivered for 2 seconds at the rate of 10 shocks/s. As can be seen in the first trace, the antidromic stimulation completely inhibited the discharge of action potentials (65 mV in amplitude) during Ringer perfusion. The decrease in steady state response, from 4.0 to 0 impulses/s, was associated with a partial hyperpolarization of the generator potential from 10 to 5 mV depolarization. After 20 min of perfusion with cimetidine (second trace), the steady state response in the absence of inhibition increased from 4.0 to 9.3 impulses/s, and the generator potential nearly doubled to ~ 20 mV depolarization. After an additional 20 min of cimetidine perfusion, the inhibitory effects of antidromic inhibition were completely abolished, the generator potential was further depolarized, and the steady state response increased to 12.5 impulses/s. Washout by Ringer perfusion partially, but not completely, reversed the effects of cimetidine before the cell was lost (data not shown).

For a molecule to be the putative transmitter of lateral inhibition, it must mimic the effects of lateral inhibition. Histamine satisfies this criterion because it decreases the rate of action potentials fired by an eccentric cell in response to light. Another criterion is that an antagonist of the putative lateral inhibitory transmitter must block its effects. The anti-histamine, cimetidine, satisfies this criterion by abolishing the inhibitory effects evoked by antidromic stimulation of the optic nerve trunk. These physiological effects of histamine and cimetidine support the hypothesis that histamine is the neurotransmitter of lateral inhibition in the *Limulus* eye.

Figure 1B also contains evidence that histamine is the mediator of self inhibition, which is a negative feedback of an eccentric cell on itself initiated by nerve impulses (6). The increase

in steady state response in the absence of inhibition is one piece of evidence. The other is the hyperpolarization of the membrane potential following the first action potential in the second trace; it represents the impulse-initiated IPSP of self inhibition. Such hyperpolarizations are minimal in trace 3 when the effects of cimetidine are maximal.

The relatively high concentrations of histamine and the long periods of treatment needed to mimic the effects of lateral inhibition are not easily explained. Contributing factors for the slow responses may include replacement volume of the recording chamber, and diffusion barriers caused by retinal tissue clogged with clotted blood. Regarding concentration, Hardie reported that 0.2 to 0.5 mM histamine was required to exert physiological effects in the fly eye (6), which is a much smaller piece of tissue than the *Limulus* eye.

The biochemical and immunocytochemical work carried out by Battelle and her colleagues shows that histamine is a major biogenic amine in the *Limulus* visual system. Histamine antibody intensely labelled cell bodies and axon collaterals of eccentric cells in the lateral eye and eccentric cell projections in the brain. Photoreceptor (reticular) cells were also labelled, but much less intensely than eccentric cells (9). Reticular cells have no known role in lateral inhibition. Rather, they transmit light-evoked current electronically to eccentric cells which then medi-

ate lateral inhibition with their neighbors (3). These results, combined with our studies with histamine and cimetidine, strongly support histamine as the transmitter of lateral inhibition in the *Limulus* lateral eye.

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Visual Performance of Horseshoe Crabs: Role of Underwater Lighting

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For millennia, horseshoe crabs have migrated toward shore during the spring to build nests and deposit eggs. Behavioral studies show that vision plays an important role in their mating behavior. Male crabs use their lateral eyes to locate mates (1). Female crabs appear to use their eyes to avoid nesting crabs (2). The visually guided behavior of male crabs is particularly striking and has been the basis for detailed analyses of the visual performance of the animal (2, 3).

Male crabs swimming along a mating beach turn and approach horseshoe crabs and objects resembling them, such as rocks, patches of seaweed, or cylindrical targets. The animal's ability to see such objects was evaluated in previous studies by measuring the distances at which males orient to targets of different size and contrast (2, 3). These studies showed that most crabs turn to hit black or grey crab-size targets (9-12" in diameter) at distances of 0.4 to 1.2 meters. Moreover, the decrease in probability of target detection with distance was nearly the same day and night. Horseshoe crabs can thus detect objects having the range of contrasts of their carapace almost equally well under a variety of lighting conditions.

We further explored the ability of horseshoe crabs to see behaviorally relevant objects using the method of two-alternative forced choice. Figure 1 illustrates the experimental setup.

During mating seasons 1995-1997, we anchored a clear Plexiglas chute to the sandy bottoms of Mashnee Dike, Mashnee, and Stage Harbor, Chatham, both in Massachusetts. The flanges of the chute guided animals toward its narrow passageway, which forced them to exit straight ahead. Upon leaving the chute, they encountered black (B) and grey (G) targets of equal size, either 9" or 12" in diameter, positioned 1 m from the exit and from each other, forming an inverted isosceles triangle. Crabs thus had the choice of turning left, right, or proceeding straight ahead. The crabs that turned either hit one of the targets, or missed to the left or right of it. We tallied the number of crabs whose behavior fell into these categories, excluding those that proceeded straight. The hit tallies of each experiment are given in Table I.

We analyzed the results by testing the null hypothesis that the black and grey targets are equally visible to horseshoe crabs. Equal numbers would then hit the two targets if animals turn left or right without preference. The probability $P(X=n)$ that n or more of N crabs respond in manner X is thus given by a binomial distribution $\beta(n, N, p)$, with p equal to 0.50. To control for directional biases caused by underwater currents, nonuniformities in the sand, shadows, and other unknown factors, we switched the locations of the two targets periodically during an

experiment. Experiments showing a statistically significant ($P < 0.05$) bias for turning left or right independent of target contrast are not included in the table. Analyzing the overall tallies, we find that equal numbers of crabs hit the black and grey targets in this visual task (202 black hits out of 395 total hits for $P < 0.34$). Equal numbers of crabs also missed the two targets (191 grey misses out of 380 total misses for $P < 0.48$) either because the animals did not see them or were unmotivated to approach them. These results are consistent with previous behavioral studies showing that horseshoe crabs can see black and grey targets almost equally well (3).

We next sorted the hit tallies into two categories according to the underwater lighting environment. We defined one category as "strobic," which included data collected on sunny days or moonlit nights when overhead waves created moving beams of light that reflected off the sandy bottom and the submerged targets (4, 5). We defined the other category as "nonstrobic," which included data collected under overcast skies or in the absence of overhead ripples or waves. We noted the strobic or nonstrobic lighting conditions as each crab left the chute because these conditions can change during an experiment, especially on partly cloudy days. The rightmost block of columns in Table I separate the hits of black and grey targets into these two categories. The columns show that greater numbers of crabs hit the grey target under strobic conditions (115 grey hits out of 199 total hits for $P < 0.017$) and the black target under nonstrobic conditions (118 black hits out of 196 total hits for $P < 0.003$). The animals' preference for the black target under nonstrobic conditions indicates that wave-induced glitter helps horseshoe crabs detect the grey target. The reversal of their target preference under strobic conditions was unexpected and may reflect a natural bias of horseshoe crabs for bright flickering objects. Indeed, their lateral eyes are maximally sensitive to light flickering at the characteristic frequencies (2–6 Hz) of wave-induced glitter in their natural habitat (6). Another possi-

Table I

Number of crabs that hit the black (B) and grey (G) targets in each experiment during the 1995–97 mating seasons. Experiments showing a statistically significant bias for turning left or right independent of target contrast are not included. In the rightmost block of columns, hits were sorted into "strobic" and "nonstrobic" categories based on the underwater lighting conditions during an experiment. These categories indicate that wave-induced underwater glitter was present (strobic) or absent (nonstrobic) as crabs exited the chute

Exp	Time	Black B	Grey G	Strobic		Nonstrobic	
				B	G	B	G
060495	night	13	1			13	1
060595	night	13	10	13	10		
061295	dusk	5	4			5	4
052896	day	1	1	1	1		
053096	dusk	9	5			9	5
053196	dusk	6	9	6	9		
060196	night	6	20	6	20		
060296	night	8	7	8	7		
060396	night	10	11	2	9	8	2
060396	day	9	6			9	6
060596	day	11	9	4	8	7	1
060696	day	11	16	11	16		
060896	day	9	7			9	7
060996	day	19	18			19	18
052997	day	0	4			0	4
053197	day	17	6			17	6
060197	dusk	11	7			11	7
060297	dusk	1	5			1	5
061597	night	17	15	17	15		
061697	night	10	12			10	12
062397	night	16	20	16	20		
overall tallies		202	193	84	115	118	78

bility is that the bright light reflected off the grey target gives it more contrast than the black one.

We conclude that the visual performance of horseshoe crabs depends strongly on underwater lighting. Strobic light created by waves on sunny days and moonlit nights enhances the visibility of low-contrast objects that are otherwise difficult for crabs to see. More animals are thus attracted to high-contrast objects in the absence of wave-induced flicker and to low-contrast ones in its presence. The visual system of horseshoe crabs and other marine animals appears highly sensitive to this prominent feature of their visual environment (4–7). Strobic underwater lighting may play an important role in their visual ecology as well.

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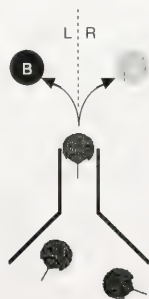


Figure 1. Horseshoe crabs swimming near the water's edge were guided into a transparent Plexiglas chute weighted to the sandy bottom. Upon leaving the chute, horseshoe crabs encountered black (B) and grey (G) cylindrical targets of the same diameter (either 9" or 12"). The targets were positioned 1 m from the exit of the chute and from each other. Crabs could thus turn either left (L) or right (R). After turning, they either hit or missed one of the targets which were switched periodically during an experiment.

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Squids (*Loligo pealei* and *Euprymna scolopes*) Can Exhibit Polarized Light Patterns Produced by Their Skin

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Cephalopods can see polarized light (1–3) and may use this sensory capacity for object detection and recognition (1, 4). Only recently was it discovered that the light reflected from the skin of cuttlefish, *Sepia officinalis* (5), and possibly octopus (6) is partially linearly polarized (termed here- “polarized reflection”), generating specific optical patterns (termed here- “polarized patterns”). In cuttlefish, the polarized patterns have been suggested to be produced by dermal reflecting cells such as those found in the “Pink iridophore arm stripes” (7). Cuttlefish are diurnal animals that interact within small groups, and the polarized patterns may be playing a part in intraspecific communication (5). Therefore, we were interested in examining the polarized patterns of other cephalopod species, namely *Euprymna scolopes* and *Loligo pealei*, that possess similar skin structures, but have a different social structure and time of activity.

The Hawaiian sepiolid *Euprymna scolopes* is a predominantly nocturnal, solitary predator (7, 8). Mating occurs at night, and apart from this brief event, the animals do not seem to engage in complex social interactions. The long-finned squid *Loligo pealei* is active day and night. It exhibits a complex range of social interactions, accompanied by various body pattern displays (7).

Sexually mature animals were examined with an imaging polarimeter capable of measuring the intensity, partial polarization (also termed “percent polarization”), and direction of linear polarization (also termed “plane of polarization,” “orientation of polarization” and “e-vector orientation”) at each pixel in an image (9, 10). In summary, the polarimeter consists of two twisted nematic liquid crystals (TNLCs; provided by the Liquid Crystals Institute, Kent State Univ., Ohio) placed in series with a linear polarizing filter (Polaroid, HN38S, serving as an analyzer) fixed at the horizontal (0°) orientation. When TNLCs are relaxed, they rotate the direction of polarization of light by a predetermined angle. When electric current is applied, the molecules within the crystals reorient and no longer rotate the light's direction of polarization. By using one TNLC set to produce a 90° rotation, and a second for a 45° rotation, the direction of polarization of the incoming light is rotated by 0°, 45°, and 90°. The overall effect is similar to rotating the analyzer to these positions. Additional technical details can be found in references 9–12. Each transmitted image is recorded with a Hi-

8 video recorder. Images (single fields) taken from the green video channel are digitized by a frame grabber and sent to a computer where they are analyzed pixel-by-pixel. From consecutive images collected at three settings of the TNLCs, we calculate the partial polarization and direction of polarization. The sensor is checked and calibrated by examining a radial polarization filter (Oriol # 25328) in air and submerged in the experimental tank. Animals were examined through a glass window, verified not to create polarization aberrations from various angles and under different illumination conditions. In all cases, the sensor was set so that the TNLCs were parallel to the glass window.

Both *E. scolopes* and *L. pealei* exhibited polarized reflection during all measurements (Fig. 1). Polarized reflection with a partial linear polarization of up to 0.8 (80%, Fig. 1B) was measured from all body parts of *E. scolopes*, but no specific pattern could be identified (Figs. 1B, C). This polarized reflection did not change with the animal's behavior. *L. pealei* presented polarized reflection at the location of the iridescent stripes in the center of its arms (Figs. 1E, F). Partial polarization here reached 0.75 (Fig. 1E). The direction of polarization was predominately horizontal (0°; Fig. 1F); however, in several cases, the direction of this polarized reflection changed by up to 30° within 1 s, without the animal exhibiting any movement or change in coloration, nor any detectable changes in the light regime in the tank. Unlike cuttlefish (5), the polarized reflection of *L. pealei* varied between the arms. In general, when a squid was sitting calmly on the bottom, polarization was strongest from the first pair of arms. While the squid was in the head-down posture, all arms showed a polarized pattern, with maximal polarized reflection recorded from the third pair. We do not know whether these changes arise from structural changes in the skin structures controlling the polarized reflection, or from specific directionality in the polarized reflection. Other areas on the squid that reflected partially linearly polarized light were the “Dorsal iridophore splotches” on the mantle, but this reflection had partial polarization of less than 0.5.

Like other cephalopods, polarized reflections are part of the body patterning repertoire of *L. pealei* and *E. scolopes*. Like cuttlefish, the polarized reflections of the social long-finned squid *L. pealei* consist of distinctive patterns that can change rapidly. The solitary *E. scolopes* exhibits polarized reflection

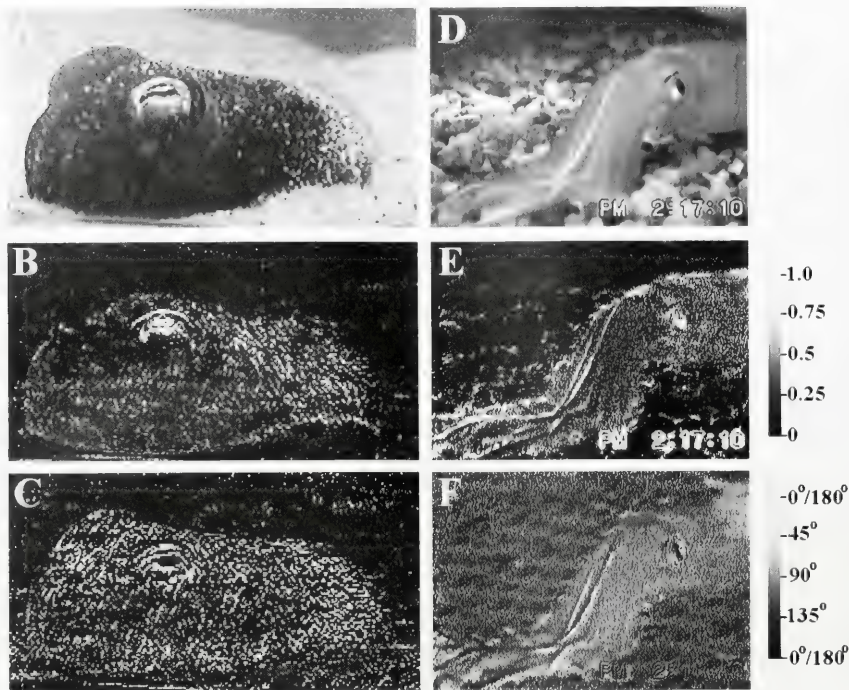


Figure 1. Intensity (A,D), partial polarization (B, E) and direction of polarization (C, F) images of *Euprymna scolopes* (A–C) and *Lolligo pealei* (D–F). Partial polarization, defined as the ratio of the intensity of the linearly polarized component to the total intensity of the light beam, ranges from 0 to 1 (0–100%) and is coded into a grey scale where black represents depolarized light and white codes for full linear polarization. Direction of polarization, ranging from 0° to 180° in the counter-clockwise orientation, is coded such that black or white represent 0°/180° (horizontal polarization) and 50% grey codes for the 90° direction, which is vertical polarization.

that appears unrelated to the animal's behavior. The functions of these polarized reflections as well as the roles of these species' polarization sensitivity (2) are yet to be determined.

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Photoreceptor Morphology and Visual Pigment Content in the Retina of the Common White Sucker (*Catostomus commersoni*)

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In addition to rods, the retina of the common white sucker is composed of single cones of variable size and unusual double cones (DCs) with unique morphological features among teleosts (1). In histological sections, the accessory member of a DC

often separates from the principal member at the level of the cone myoid. In other fishes, the separation typically occurs in the more distal ellipsoid region of the receptors' inner segments. Furthermore, in tangential sections, the apposing membranes of

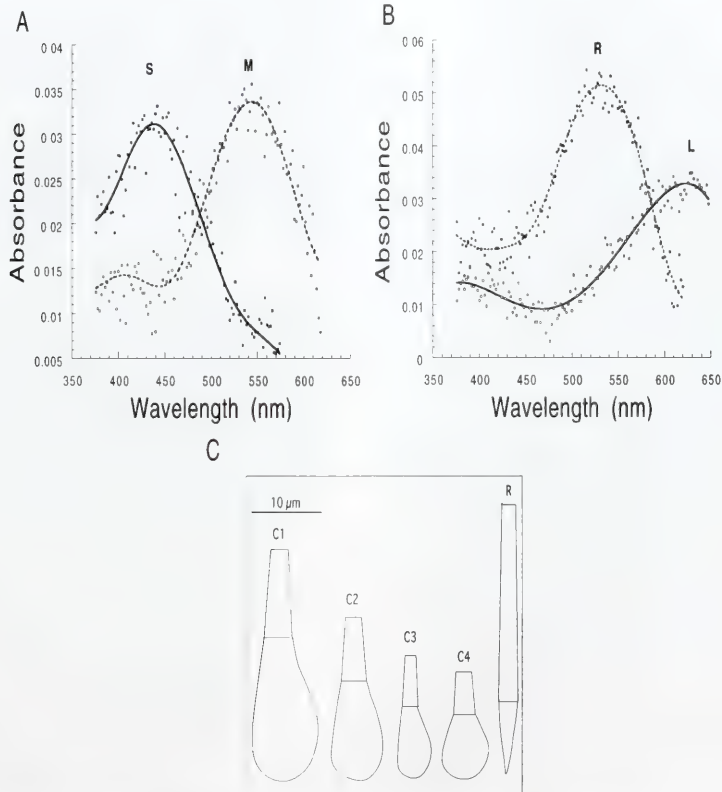


Figure 1. Visual pigments and photoreceptors in the common white sucker. (A, B) Average spectral absorbances of the blue or short (S) wavelength sensitive cones, the green or middle (M) wavelength sensitive cones, the rods (R), and the red or long (L) wavelength sensitive cones. The solid curves were obtained by Fourier filtering the experimental data (2). The transverse specific densities (in μm^{-1}) for the rod, and the S, M, and L cones were 0.0118, 0.0132, 0.0112, and 0.0101, respectively. These values are similar to those obtained for goldfish (5). The total number of photoreceptors examined were: 10 (S cone), 17 (M cone), 34 (L cone), and 7 rods. (C) Drawings show video image traces of the four cone types observed (C1–4) and the rod (R). C4 was rarely seen and we failed to obtain absorbance recordings from this type of cone, which may contain the ultraviolet sensitive pigment. The average total length and weight of the fish studied ($\pm$ SD) were: 19.8 ($\pm$ 0.66) cm and 39.05 ($\pm$ 4.1) g; n = 3 fish, 6 retinas.

the two cells frequently appear far apart, and the gap is filled with granular material, a situation unknown in other species. In this study, we examined the absorbance properties of common white sucker photoreceptors in an attempt to correlate form and visual pigment content in this unusual retina.

Pieces of retina extracted from dark-adapted animals were teased apart in Ringer solution, placed between two coverslips, sealed, and individual photoreceptors examined with the dichroic microspectrophotometer (2). The sample measurements consisted of placing a beam of $0.6 \times 2 = 1.2 \mu\text{m}^2$ cross section on a photoreceptor outer segment along its length and taking an average of eight consecutive transmission scans from 270 to 648 nm. Reference measurements were also recorded from adjacent areas devoid of tissue to compute photoreceptor absorbance (2). Video images of the preparations showed the shape and dimensions of photoreceptors.

We found three spectral classes of cones and one rod type in the retina of the common white sucker (Figs. 1A, B). Although optic nerve recordings from anaesthetized live fish have suggested that cones with maximum absorbance in the ultraviolet should also be present in this species (1), we did not detect any such cell by microspectrophotometry (MSP). The cone outer segments had average absorbance maxima in the blue (439 nm), green (544 nm), and red (~ 630 nm) regions of the spectrum, while the rod absorption peaked in the green (536 nm). The long wavelength peak of the red pigment and the large spectral bandwidths at half maxima for the measured cones and the rod ($\sim 4700 \text{ cm}^{-1}$) suggest that this fish possesses a combined vitamin A_1/A_2 retina containing primarily vitamin A_2 [a bandwidth value of 4800 cm^{-1} is expected for a pure vitamin A_2 -based visual pigment with maximum absorbance in the green spectral range (3)]. The red cone maxima of the common white sucker, in fact, approximates that of the longest wavelength-absorbing red pigment ever found in a vertebrate, the piranha, at $630 \pm 5 \text{ nm}$ (4).

There was no correlation between cone morphology and visual pigment content; i.e., all the spectral classes were found for all the cone types examined (C1–3, Fig. 1C). Interestingly, paired cones were absent, even though green and, especially, red-ab-

sorbing cones were abundant. Using the same preparation method, MSP investigations on retinas from cyprinid fishes, a group closely related to the suckers, have yielded DCs in large numbers that were red/green pairs (5, 6). The absence of paired cones in our preparations and the presence of single red and green cones suggest that the DC pairs observed histologically (1) became separated in this study. Such separation is unknown for DCs previously examined by MSP and thus leads us to question a close functional connection between common white sucker cone pairs.

Unlike other teleosts that are sensitive to ultraviolet light and possess paired cones, the common white sucker is unable to discriminate polarization (1). This result would be expected if the paired cones are uncoupled (as suggested by our MSP observations) and each cone acted as a single circular waveguide. Indeed, a previous study suggests that the dividing partition of DCs can provide the optical anisotropy for polarization detection (7). However, in contrast with the regular square mosaic patterns present in other teleosts, the cones in the centro-temporal retina of the common white sucker are arranged randomly (1). If the centro-temporal retina is a dedicated polarization detection area in polarization sensitive teleosts (1), and if a regular mosaic is required for polarized light discrimination (1), then both the lack of ordered photoreceptors and closely apposed DCs is consistent with the lack of polarization sensitivity previously observed for the common white sucker.

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Chemosensory Search Behavior in the Starfish *Asterias forbesi*

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Starfish, like many animals, rely heavily on chemoreception. Their radially symmetric body plan and decentralized nervous system suggest that they use different chemosensory search strategies than other animals. Starfish chemosensory behavior has not been well studied, most researchers reporting orientation success rates but not paths or orientation strategies. However, orientation behavior has been studied in *Asterias forbesi* under

no-flow conditions (1) and, by the author, in a y-maze to observe success rates and ray choice (2). Although y-maze experiments adequately test success rates, they are unsuitable for studying path parameters because of wall encounters. In no-flow experiments, odor delivery is unpredictable, although these do eliminate concerns about rheotactic behavior—starfish have been variously reported to move upstream (3) or cross-stream (4)

Table 1

Starfish path parameters

	t-test			Regression			
	Mean	N	Signif. ¹	df	Slope (β)	r ² (%)	Signif.
Movement Rate (cm/min)							
Orienting	13.16	12	$P < 0.001$	267	0.316	<1	NS
Non-orienting	15.48	12	NS	355	0.008	<1	NS
Control	14.57	10	$P < 0.01$	293	0.007	<1	NS
Heading Angle (°)							
Orienting	35.7	12	$P < 0.001$	267	0.584	25.8	$P < 0.001$
Non-orienting	92.0	12	NS	355	0.349	4.0	$P < 0.001$
Control	87.4	10	$P < 0.001$	293	0.38	5.4	$P < 0.001$
Turning Angle (°)							
Orienting	28.8	12	$P < 0.005$	255	0.086	1.4	$P < 0.05$
Non-orienting	36.3	12	$P < 0.005$	342	-0.086	0.8	$P < 0.05$
Control	29.8	10	NS	278	0.024	<1	NS

¹ The first row in each block lists orienting vs. non-orienting, the second lists non-orienting vs. control, and the third lists control vs. orienting.

when in flow with no odor. To determine path parameters of orienting starfish in a known stimulus environment, chemosensory search behavior was observed in a large (12-m by 2-m) recirculating flume. To test for rheotactic behavior, additional starfish were observed under the same flow conditions without odor.

Starfish were collected by divers and maintained in circulating seawater aquaria. Starfish were fed mussels but starved at least 1 week before trials. Orientation trials were carried out in a recirculating flume 12 m long and 2 m wide. The mean stream-flow velocity was 5 cm/s, but at the height of the odor source (2 cm above the bottom) flow slowed to 3 cm/s because of boundary-layer effects. The odor source was 5 g/l mussel tissue in seawater, dyed with rhodamine for visibility. During trials, starfish were picked up—by holding them as centrally as possible to avoid biasing initial movement direction—and placed in the odor plume at about 1 m from the source.

Trials were videotaped and digitized to determine location and orientation every 30 s. From these digitized paths, four orientation parameters were extracted. Movement rate is the speed at which the starfish moved. Heading angle is the angle between the direction of movement and the direction from the starfish to the odor source: heading of 0° indicates movement directly towards the source. Turning angle is the change in the direction of movement. These definitions conform to those of Moore *et al.* (5). Rotation angle is the change in absolute body facing. Being radially symmetric, starfish can change body facing and heading angle independently.

For comparison, trials were classified into three groups. A starfish was categorized as orienting if it moved into a 15-cm-square target area at the odor source within a 15-min trial period and as non-orienting if it did not enter the target area within the time limit. Control trials involved no odor stimulus. No control starfish met the criterion for orienting.

About 20% (12) of the starfish oriented to the source in the allotted time. This low rate most likely reflects insufficient motivation to orient; others have reported that a 2-month starva-

tion period before trials results in more consistent behavior (4). The behavior of orienting starfish was markedly different from that of non-orienting ones. Non-orienting starfish typically moved in exploratory patterns, making little net progress and often circling or otherwise recrossing their paths. None moved as far upstream as the target area, even outside the plume, and only one reached the side of the flume. In contrast, orienting starfish always moved directly towards the source (in two cases only after an initial circle), staying within the odor plume. Control starfish behaved much like non-orienting starfish. One control animal moved the full upstream distance but missed the target area; others circled (six) or moved generally upstream (one) or downstream (two) without reaching the extreme ends of the experimental area.

All 12 orienting paths, the first 12 non-orienting paths, and 10 control paths were analyzed. Some starfish in the orienting and non-orienting groups may have been used in two trials, but the statistical tests used are sufficiently robust to allow for this possibility. Path parameters were analyzed with a *t*-test suitable for unequal sample sizes for differences between the three groups (orienting, non-orienting, and control) and by regression analysis against distance to the source (Table 1). Rotation angles are not listed; mean rotation angles were 10°–11.5° and not significantly different from one another; nor were they correlated with source distance.

Heading angles were lowest in orienting starfish. Heading angles of orienting starfish decreased as they approached the source; this effect was reduced in non-orienting and control starfish. Decreasing heading angles in orienting animals are indicative of a chemotactic orientation strategy rather than a rheotactic strategy (5). Other orientation experiments with *Asterias forbesi* have also suggested chemotaxis (1,2) and decreased heading angles with approach to the odor source (1).

Movement rates were also lowest in orienting starfish. In controls, movement rates were correlated with time, increasing over the course of trials ($r^2 = 26.6\%$, $P < 0.001$, $\beta = 2.01 \times 10^{-4}$ cm/s²), whereas there was no correlation in orient-

ing starfish and a smaller correlation in non-orienting starfish ($r^2 = 4.7\%$, $P < 0.001$, $\beta = 8.4 \times 10^5$). Starfish typically cease movement after handling and then gradually return to normal movement speeds over several minutes. However, the lack of normal response in orienting starfish, paired with lower mean movement rates, suggests that decreased speed is characteristic of orientation behavior. Lower movement rates may improve the ability of starfish to make accurate comparisons of odor concentrations.

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Behavioral Dynamics That Would Lead to Multiple Paternity within Egg Capsules of the Squid *Loligo pealei*

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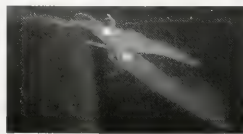
Studies of sexual selection are important for advances in evolutionary theory. They also provide clues to help manage populations of commercial species. The long-finned squid *Loligo pealei* is a valuable resource (1) that is fished mainly on spawning grounds. A central question is how such targeted fishing might affect the species' reproductive behavior. One possible consequence would be that a particular fishing method removes, for example, the largest, fittest males, so that genetic recruitment would be affected directly. Because *L. pealei* has a brief one-year life cycle (2), the deleterious effects of such a scenario would manifest themselves quickly.

Our hypothesis is that the mating system of *L. pealei* is robust and flexible, and that there is a high degree of genetic mixing, not only at communal egg beds, but within the individual egg capsules. We are currently testing this hypothesis through behavioral studies. Previously, Hanlon (3) identified some of the principal behaviors on the spawning grounds, including those of the "sneaker" males, and noted the high potential for multiple paternity within egg capsules. We now report new findings from field observations during May 1997 (6 h of videotape from 22 SCUBA dives in Vineyard Sound), supplemented with 130 h of laboratory trials conducted from May through August. Squids used in the laboratory trials were jig-caught and were studied over several weeks.

Field observations suggest at least four behaviors through which males of various sizes gain copulations with females. First, a large male may pair temporarily with a female, mate her, and guard her from rival males before, during, and after mating and oviposition events. On spawning grounds, females are rarely unpaired for any length of time, so lone large males must first supplant a paired male before pairing with the female. Second, a lone large male sometimes foregoes the fighting, guarding, and pairing phases and quickly grabs a paired female

and copulates with her; this behavior has been recorded when the female is very close to the egg beds (Video 1). Third, small males commonly act as "surreptitious" sneakers by remaining at a distance from the egg beds, then rapidly jetting towards females when her large consort male is not vigilant, mating her, and escaping rapidly. Fourth, small males may act as "bold" sneakers by swimming near the egg beds and intercepting pairs that approach the beds. Of these male behaviors, the second and fourth were observed for the first time in *L. pealei* during 1997.

Pairing with a female confers at least two benefits for males at the spawning grounds: copulation success and an "owner advantage" in fights. Males paired with a female were successful in 30 of 34 observed copulation attempts (88%). In contrast,



<http://www.mbl.edu/html/BB/homeBB.html>

Video 1. Video sequence of an extra-pair copulation by a large lone male. At the start of the segment, the female is in the center of the screen, just to the right of the egg bed. Her paired male is just above her. As she begins to place her arms into the bed, a lone, large male darts in from the left, grabs her mantle, and proceeds to copulate in the parallel position. After 14 s of copulation, the female's paired male starts to wedge himself between her and the copulating male. Nevertheless, the copulation lasts for a total of 28 seconds, which is a typical duration for parallel copulations. This video can be viewed at the URL listed above.

unpaired males that were longer than the female were successful in only 10 of 38 attempts (26%), and unpaired males that were of the same mantle length as the female were successful in 13 of 64 attempts (20%). This lower copulatory success for unpaired males might reflect female choice; indeed, we have observed females jet from males that attempt to copulate in the field and laboratory. With regard to agonistic bouts at the spawning grounds, paired males remained with the female when challenged by another male in 23 of 24 contests. This high fighting success of paired males might explain the alternative large male behavior; *i.e.*, repeatedly defeated lone large males might resort to a form of "sneaky" mating.

At the spawning grounds, only large males behaved as paired consorts to females, which were always smaller (mean $\pm$ SE mantle length of females jigged in May: 17.2 ± 0.4 cm, $n = 26$). In the laboratory, a similar trend was observed in six trials that involved four males (two large males, *i.e.*, mantle length greater than 17 cm, and two small males, *i.e.*, mantle length less than or equal to 17 cm) and two females. We conducted scan surveys every 10 min. At each scan, a male was considered to have been guarding a female if he was swimming within two body lengths of her and was actively positioning himself between her and the other males. In these trials, only one female was being guarded during a given scan. Of 72 scans, one of the largest two males was guarding a female in 62 scans, whereas one of the shortest two males was guarding in 10 scans (χ^2 with Yates correction = 36.13, $P < 0.0001$).

This result indicates behavioral flexibility among males in the laboratory. In particular, a few small males guarded females in the presence of larger males. This might indicate motivational differences between males that become more evident when squid are confined. That is, some large males might have opted not to compete for females in the trials, thereby creating the opportunity for more highly motivated small males to pair with females. In contrast, large squids at the communal egg beds are probably highly motivated to reproduce and thus displace any small males that might attempt to guard females. As in the laboratory, motivational differences might exist between males in the field. Some reproductively inactive squids can usually be seen several meters from the egg beds, suggesting that some of them might have temporarily dropped out of the competition for females.

Behavioral flexibility in mating position has also been documented. Two copulatory positions are known: parallel and head-to-head (4,5). In a field video subsample, large males attempted 14 parallel copulations and one head-to-head. In contrast, small males attempted 9 head-to-head and 9 parallel copulations. Long-term focal observations of 10 males in the laboratory (each male attempting 4–40 copulations) demonstrate that small as well as large males (mantle length range: 14–27 cm) will attempt to copulate in both positions within the same day.

Sperm are placed in different locations in the two mating positions. In parallel copulations, the male places one or more spermatophores near the female's oviduct. In head-to-head copulations, one or more spermatophores are attached to, and eventually transferred into, the female's seminal receptacle (6). During oviposition, the eggs pass out of the oviduct and into the mantle cavity where, presumably, there are swarms of swim-

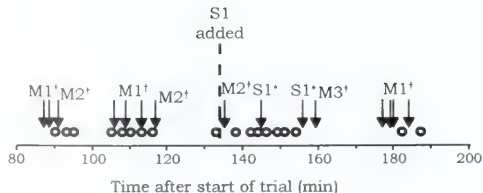


Figure 2. Multiple copulations and oviposition in a laboratory trial. This trial started when two males and a female were added to a tank already containing a male and female (the "resident" squid). M1: resident large male, M2: introduced large male, M3: introduced large male, S1: introduced small male. Each open circle represents the oviposition of one egg capsule by the resident female (she laid 18 capsules in this trial). Arrows represent copulations by males, where ^q signifies head-to-head copulation, ^p parallel copulation.

ming sperm (from a male parallel mating). The eggs are then drawn out through the funnel and into the female's arms. How sperm in the seminal receptacle (just below the mouth) are released or controlled by the female is unclear, but she probably has the option of releasing stored sperm while holding the egg capsule in her arms before depositing it on the substrate. This suggests the possibility of female choice over potential sires at this late stage.

Females mate with multiple partners, and many different sequences can occur. In the field, for example, we observed females mating with two or more consecutive males. In the laboratory, we saw four sequences of mating and oviposition in small, mixed-sex groups of squid. Each sequence involved one female who mated with two or more males during several hours of oviposition. In some cases, as many as three different males copulated immediately before the laying of a single egg capsule (Fig. 1). Given such opportunities for competition between the sperm of potential sires, the question of relative fertilization success among males becomes crucial. We are currently developing DNA markers (microsatellite loci and RAPD) to assess the paternity of these offspring (7).

In summary, these new observations document several male mating behaviors. Males may temporarily pair with females; males in such pairs experience high copulatory and fighting success. Alternatively, males may employ one of several "sneaker" tactics. Copulation involves one of two positions, each of which involves a distinct location of sperm placement. In addition to these behavioral differences between males, individual males can copulate in both positions, even within the same day. The social dynamics of *L. pealei* suggest a high level of genetic mixing in communal egg beds, as well as within individual egg capsules.

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Reference: *Biol. Bull.* **193**: 214–215. (October, 1997)

Far Field Chemo-orientation in the American Lobster, *Homarus americanus*: Effects of Unilateral Ablation and Lesioning of the Lateral Antennule

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American lobsters (*Homarus americanus*) use their paired lateral antennules to locate distant odor sources, such as those from food and mates (1,2,3). American lobsters require the normal function of both antennules for normal near field (<60 cm) chemo-orientation (3) and respond differentially to chemical signals applied to one antennule but not the other (4). The latter study determined the effects of the ablation of an entire lateral antennule (containing both mechano- and chemo-receptors). In the present study, we have examined the effect of selectively destroying chemoreceptors alone. We also determined the effect of receptor loss in a far field (3 m) odor environment, which we have previously shown in studies of turbulent odor dispersal to be a significantly different stimulus environment from the near field (1,5,6,7).

Twenty-three female lobsters were randomly assigned to one of three "treatment" groups: (A) "Shams" ($n = 9$) were handled in a similar manner to the other groups, but were otherwise left intact; (B) "Lesions" ($n = 7$) had their right lateral antennule dipped in deionized water for 5 min. The reduced osmotic pressure lyses the dendrites of chemoreceptors (8,9) while sparing the mechanoreceptors on the antennule; and (C) "Ablates" ($n = 7$) had their right lateral antennule cut off at the base.

Orientation trials were performed in a recirculating flume ($14.5 \times 3 \times 1$ m), with the most downstream 3 meters serving as the behavioral testing arena. At the upstream end of this arena, a jet of filtered tuna puree in seawater [0.75% (w/v)] was injected into a constant background flow of ~ 4.5 cm/s. The downstream end of the arena contained a centrally located shelter, into which the lobsters readily settled. Their behavior was recorded by three video cameras, with overlapping fields of view, mounted 2 m over the arena. In all trials, we recorded the behavior of each lobster for 10 min before turning the stimulus on, and then for 10 min while the stimulus was injected into the background flow. For each animal, a "pretreatment" trial

was performed prior to "treatment" trials. Four "posttreatment" trials were performed, at intervals of 0, 1, 3, and 5 days, respectively. The data from posttreatment trials were pooled.

The number of instances where lesioned or ablated lobsters contacted the source was too small for statistical analysis across all groups. Therefore, a statistical analysis of near field orientation behavior was not performed. However, an analysis of behavioral events in the far field portion of the odor plume was possible. First, the number of trials during which the lobsters left the shelter in both the pretreatment and posttreatment conditions was scored. Then, two different methods were used to score the initial direction taken by the animal as it left the shelter, with the shelter as a point of origin: (A) The "initial direction" of the lobster was defined as either "source-directed" ($\pm 30^\circ$ to the right or left of the origin) or "non-source-directed" ($> 30^\circ$ from the origin); and (B) The "heading" of

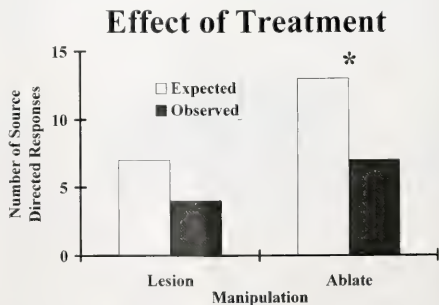


Figure 1. Effect of treatment on the number of source-directed responses in *Homarus americanus*. White bars indicate number of responses expected from the behavior of animals in the sham treatment group. Black bars indicate actual number of responses in both the lesioned and the ablated treatment groups. Asterisk indicates significant difference between ablated and sham treated animals ($P < 0.02$).

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the lobster was defined as the angle between the path of the lobster from the shelter to its position 30 cm upstream, and a direct path from the shelter to the source.

Sham treated animals emerged from the shelter significantly less often when the stimulus was injected into the background flow than in the absence of a stimulus [$\chi^2(1,71) = 13.14$; $P < 0.01$]. Lesioned animals left the shelter significantly less frequently in the presence of the stimulus [$\chi^2(1,55) = 29.94$; $P < 0.01$]. Ablates showed no significant differences. In trials where the animals left the shelter, shams were initially "source directed" ($\approx 30^\circ$) significantly more often than in the premanipulation trials [$\chi^2(1,20) = 7.32$; $P < 0.05$]. Lesioned animals did not differ significantly from shams in the number of source-directed responses during trials where stimulus was present. Ablates, however, exhibited significantly fewer source-directed responses than shams in this condition [$\chi^2(1,26) = 5.45$, $P < 0.02$] (Fig. 1).

Our results demonstrate that the loss of an entire antennule interferes with the ability of American lobsters to orient to a far field odor source. We did not find a significant difference in the behavior of animals deprived of only chemoreceptors on one lateral antennule. This may suggest that a sense of flow on both antennules and chemoreception on one is sufficient for normal chemo-orientation. However, in view of the significant depression of overall orientation responses in the lesion treatment group (with stimulus), it may be premature to draw this conclusion from a negative result without further experimentation.

This study points to the central role of the lateral antennules and bilateral information processing in *far field* chemo-orienta-

tion in the American lobster. Devine and Atema (3) found that in the near field (<60 cm) odor environment, unilaterally ablated American lobsters make a significantly greater number of incorrect initial turns when presented with a choice between a flavored or unflavored stimulus jet. Our results demonstrate that both the antennules are necessary for a lobster to orient normally when it is sampling in the far more temporally and spatially variable far field odor stimulus environment (1,4,6,7).

We thank Christina Manbeck, Dr. David MacMillan, and Dr. Rainer Voigt for their advice and technical assistance in the execution of this project. This work was supported by an REU supplement to NSF grant IBN-9222774 to JA.

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Effectiveness of Continuous Bilateral Sampling for Robot Chemotaxis in a Turbulent Odor Plume: Implications for Lobster Chemo-orientation

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Many animals have paired, spatially separated sensors that locate stimulus sources in space; interaural time-of-arrival difference estimation in audition, and stereopsis in vision, are well known examples. Like many invertebrates, the American lobster, *Homarus americanus*, possesses paired external chemosensor organs (the lateral antennules). This lobster in particular appears to require both of its antennules for efficient chemical source localization (1), which suggests that it might employ something like stereopsis or interaural time-of-arrival difference on chemosensory signals for guidance to a chemical source. A simpler hypothesis is that the lobster uses its two antennules to estimate local concentration gradients.

Direct tests of this hypothesis are complicated by the difficulties of controlling chemical dispersal patterns over the 1–

10-m scale appropriate for lobster chemo-orientation, and by the other sensory modalities that provide the lobster with guidance information. In this study we avoid the first problem by using a "standard plume" that has been well characterized statistically (2,3,4) and that lobsters orient toward (5,6). As a model for the lobster we use an autonomous underwater robot (7,8), which derives its sole guidance from chemical concentration signals. The robot's success at locating odor sources supports the notion that chemo-orientation is possible for a lobster relying solely on two chemical sensors for guidance.

With a 3 cm inter-sensor separation, robot orientation is effective when the robot starts 60 cm downstream from the source, but not when it starts at 100 cm (8,9). One might expect that increased sensor separation would produce improved perfor-

mance. In this study we examined the effectiveness of four different sensor separations (1, 3, 5, and 7 cm) at two starting distances (60 and 100 cm), directly downstream from the source. Twelve 30-s trials were run in each condition. The algorithm [described in (8)] steers the robot toward higher concentrations, and backs the robot up when it detects an absence of signal on both sensors. The former rule provides guidance, the latter keeps the robot in the plume. The plume source is a jet of neutrally buoyant aqueous 0.76 M NaCl and ethanol injected into a flow-through, fresh water flume ($3 \times 1 \times 0.5$ m, background flow ~ 1 cm/s). The sensors detect salt concentration as conductivity (8.9,10), which is sampled at 25 Hz. The sensor signals are averaged into 0.5 s, which is the approximate time scale of the lobster's peripheral olfactory processing (11). The robot's size, speed, and turning are scaled to that of the lobster.

Starting 100 cm downstream, the robot did not reach the source regardless of sensor separation. But starting at 60 cm downstream, the number of "hits" increased with sensor separation: once each at 1 and 3 cm, five times at 5 cm, and nine times at 7 cm. The robot paths produced at the 60- and 100-cm starting distances were markedly different. The latter are irregular, with many abrupt turns and back-ups, reflecting the discontinuity of the plume at this distance, while the former (60 cm) contains smooth turns, alternating left and right, and leading to the source. Path tortuosity (change in turning angle) increased with sensor separation [$F(3,1,87) = 8.73$ $P < 0.001$] but was not significantly affected by starting distance. The robot traveled significantly shorter total distances per trial when starting at 100 cm compared with 60 cm [$F(3,1,87) = 11.80$ $P < 0.001$], presumably because the greater signal intermittency downstream occasioned frequent shifts from forward to reverse motion. Increased sensor separation did not improve in closest approach from this starting location.

By definition, spatial gradients increase with distance. Therefore, increased sensor separation should afford the robot better signal-to-noise ratios for gradient estimation and steering toward the source. This was observed at the 60-cm, but not at the 100-cm starting distance. At 60 cm, the plume is narrow and the contrast between plume and background water is therefore high at the plume borders. Increasing the sensor separation makes detecting this gradient easier by increasing the probability of one sensor being in and the other out of the plume. The failure of the

robot to make significant progress toward the source from the 100-cm starting position (where the plume is much wider) indicates that increasing the sensor separation beyond the ~ 3 cm separation of lobster lateral antennules does not provide an improved estimate of the local concentration gradient.

We conclude that the lobster must use a strategy different from instantaneous concentration-gradient estimation for guidance at distances greater than 1 m. Animals using this strategy on the lobster time scale require inter-sensor separations greater than 7 cm (probably close to the plume width) for effective orientation between 1–10 m downstream from the source. This conclusion leaves open the possibilities that the lobster is using non-chemical cues (e.g., chemically gated rheotaxis) or employing memory of recent signal trends for guidance. Also, the lobster's decreased performance when deprived of one antennule (1), taken together with our results, suggests that this animal may be using a more sophisticated bilateral comparison (e.g., interaural time-of-arrival difference estimation) method than instantaneous concentration-gradient estimation.

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Can the Female American Lobster Predict the Dominant Male?

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The male American lobster, *Homarus americanus*, is a solitary animal that shares its shelter only during times of mating (1,2). Mating takes place after molting of the female lobster. The female is vulnerable at this time because of her soft exoskel-

eton, and for protection she cohabitates with a male. Because a male can be evicted by a more dominant male, she must choose her mate carefully. Studies suggest that chemical signals carried in the urine play a role in mating behavior (3,4). In a Y-maze choice test, female American lobsters preferred the dominant over the subordinate male (5). In those tests the males had lived together and had established a permanent dominance

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hierarchy. It remains unclear whether a female lobster can predict which of two unfamiliar males will become dominant.

Lobsters (79–88 mm in carapace length) were caught locally in Woods Hole, Massachusetts. Female and male lobsters were size-matched within 3 mm for each group and placed in isolation tanks (10 l) for 10 days prior to an experiment. Experiments were run in a $3.7 \times 1.4 \times 0.3$ m fiberglass flume containing three lobsters. At the upstream end, single males occupied two shelters that were separated by a board, creating a Y-maze. A third shelter, at the downstream end, housed a single female and was exposed to water flowing through both male shelters. The entrance to each male shelter was blocked by a barrier that allowed the female to attempt entry, but kept her from entering successfully. Each day three females were tested with two different pairs of males. The tests were repeated for 10 days. Overall, 18 groups were used over a six-week period. Males were acclimated for 40 min prior to a test. The female was then placed in the downstream shelter and acclimated for 15 min before being allowed to leave her shelter. Each 15-min experimental test was followed by a 25-min break to allow water to clear the flume and to reacclimate the males after any possible female stimulation. Control groups were run with both male shelters empty. To ensure that the flow of water in the flume remained constant (~ 1.5 cm/s), the flow rate was checked every day by release of dye. The experiments were taped with an overhead video camera, and the female's path was tracked on a computer. Behaviors occurring in a 0.30×0.40 m area in front of the male shelters were recorded as first choice, side choice, entering behaviors, and time spent. After 5 days of trials, the male pairs were placed in a small tank ($60 \times 90 \times 59$ cm) and allowed to interact for 30 min; the procedure was repeated the next day to ensure that a hierarchy was well established. Lobsters that lost the first fight—i.e., avoided the winner—remembered this outcome of agonistic encounters for at least 7 days (6). Over the next 5 days, the trials were repeated between each female and the same pairs of males.

Female behaviors are summarized in Table 1. Before the male lobsters' agonistic encounters, females preferred the dominant male on the first day and then made alternating choices between the two males, with no significant preference for either male (Chi-Square test, $P > 0.05$). After dominance was established, females showed a preference for the subordinate male on the first day and then alternated their choice over the next 4 days from a slight preference for the dominant male to a slight preference for the subordinate male.

The female lobsters seem incapable of predicting the domi-

Table 1

Female shelter-related behaviors at the dominant and subordinate male shelters

Day	First choice		Mean time (s)		Side changes		Behaviors	
	Dom.	Sub.	Dom.	Sub.	Dom.	Sub.	Dom.	Sub.
1	12	6	166	53	113	76	154	34
2	8	10	111	145	59	68	75	175
3	7	7	99	120	66	63	66	44
4	7	8	115	122	66	52	87	66
5	7	8	150	58	75	49	81	49
6	4	14	131	123	83	88	78	103
7	10	5	77	102	68	49	15	42
8	7	9	110	92	52	68	109	103
9	10	6	143	129	78	65	103	28
10	8	9	98	157	63	87	129	172

nant male of a pair before and after the pair's agonistic encounter. This may indicate either that there is no chemical signal or that the signal cannot be interpreted by the American lobster. In prior studies (5) in which the female chose the dominant male significantly more often, the dominant/subordinate relationship was well established; i.e., except for the tests, the males lived together in the same tank. In this study, we could not reproduce those results: after the male agonistic encounters, females showed no consistent preference and made alternating choices. This suggests that females can determine the dominant male only if the males live together and maintain their hierarchy over a long time. Short agonistic encounters do not seem to change the chemical cues sufficiently to allow females to detect the dominant male.

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UV Cutting of MAPs-bound Microtubules

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Microtubules exhibit dynamic instability *in vivo* and *in vitro* (with purified tubulin), stochastically switching between phases of persistent growth and shortening (reviewed in ref. 1). The growth phase is governed by the presence of a tubulin GTP cap at the end of the microtubule, and the structure we observe is tubulin protofilament sheet folded up into a blunt and straight microtubule. The shortening phase is initiated by the loss of the tubulin GTP cap, and we see the blossom structure of protofilaments, curved inside-out. The transitions back and forth between the growth and shortening phases include an intermediate phase in which the microtubule has lost its tubulin GTP cap but still, transiently, retains the straight, blunt structure characteristic of the growth phase (2). Thus, for a microtubule to grow, new subunits must be added to the tubulin GTP cap; loss of the cap puts the microtubule in the intermediate phase. And for a microtubule to shorten, the protofilaments must first peel away from the central lattice and subsequently break off (3). If microtubules are polymerized with purified tubulin, cutting off the tubulin GTP cap, either mechanically or with UV, produces microtubules in the intermediate phase (2). For plus ends, the intermediate phase quickly switches to the shortening phase. For minus ends, the intermediate phase quickly switches back to the growth phase (2). Microtubule-associated-proteins (MAPs)—particularly neuronal MAPs such as MAP2 or Tau—have been shown to suppress microtubule dynamics (4). To test the hypothesis that MAPs promote switching from the intermediate to the growth phase, we used UV irradiation to cut the ends off MAPs-bound microtubules.

Sea urchin axonemes were added to a coverslip and glass slide chamber. The axonemes adhered to the surface of the coverslip. Then partially purified (*i.e.*, 2-cycles) porcine brain tubulin (1.3 mg/ml) was added to polymerize microtubules. Note that porcine brain tubulin, after 2 cycles of warm-cold purification, still retains the endogenous MAPs from the homogenate, particularly MAP2 and Tau (5). MAPs-bound microtubules were observed by video-enhanced differential interference contrast microscopy, and severed with a UV microbeam. The experimental apparatus has been described in detail (6).

Using a 5-s exposure of a UV microbeam (a 200-W mercury arc lamp served as the source), we cleanly severed single microtubules extending from the axonemes. We severed 23 minus ends and 39 plus ends. All were stable after being severed and did not exhibit immediate shortening. Instead, they continued to grow (Fig. 1).

The behavior of severed minus ends of MAPs-bound microtubules was similar to that of severed minus ends of purified-tubulin microtubules: they quickly switched from the interme-

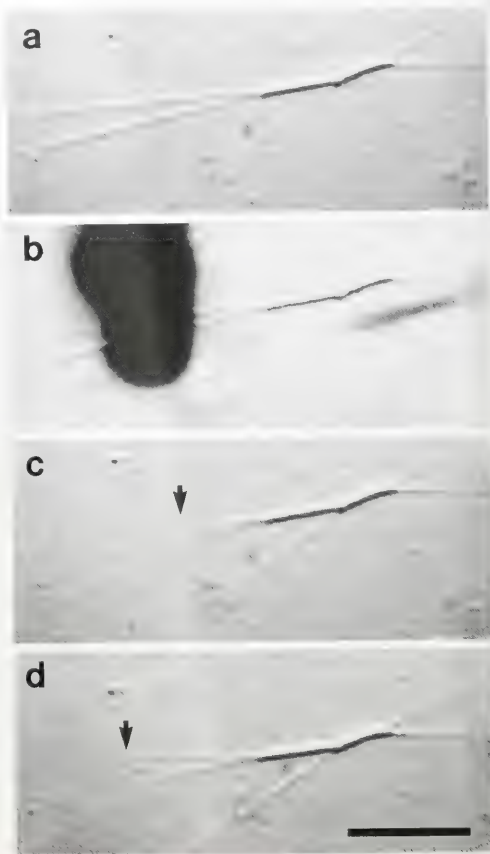


Figure 1. MAPs-bound microtubules polymerized from axoneme being cut by UV irradiation. (a) Two long plus end microtubules extending to the left from the axoneme, time = -10 s; (b) the shadow (black) of the reflecting mirror about to focus UV light onto the microtubules, $t = -1$ s; (c) after 5 s of UV exposure, the microtubules are cleanly severed, $t = 6$ s; (d) the severed plus ends do not shorten, but instead continue growing (arrow), $t = 70$ s. Scale bar: 5 μ m.

mediate phase back to the growth phase. However, the behavior of the severed plus ends of MAPs-bound microtubules was different from that of severed plus ends of purified-tubulin microtubules: the severed MAPs-bound microtubules

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switched back to the growth phase, whereas severed purified-tubulin microtubules immediately switched from the intermediate state to the shortening state. This implies that MAPs promote the transition from the intermediate to the growth phase. Perhaps through their binding to the microtubule lattice they prevent the tubulin protofilaments from the intermediate phase from splaying outward and peeling away from the microtubule lattice.

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Actin Bundles in Neuronal Growth Cone Observed with the Pol-Scope

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The neuronal growth cone appears at the tip of dendrites and axons where it plays an important role in the navigation of dendritic and axonal growth. Growth cones are rich in actin fibers, which are presumably involved in growth cone movement. Careful observations of labeled actin filaments with a fluorescent microscope or of native actin bundles with a video-enhanced differential interference contrast (DIC) microscope have revealed important aspects of actin-related dynamics, such as retrograde flow (1), treadmilling of actin (2), and the involvement of myosin V in filopodial elongation (3). But the molecular mechanisms of growth cone movement are still in debate, because actin bundles in living cells are difficult to visualize. New methodologies are needed for understanding the mechanisms of growth cone movement. Therefore, we observed actin bundles in living growth cones with the Pol-Scope (4). In contrast to the traditional polarized light microscope, the Pol-Scope reveals birefringent components independent of their orientation, and measures their retardance with high sensitivity and resolution over the whole field of view (4). In this paper, we demonstrate the feasibility of observing unlabeled actin bundles in living growth cones using the Pol-Scope and compare Pol-Scope image with DIC image.

Bag cell neurons, from the abdominal ganglion of the marine opisthobranch mollusc, *Aplysia*, form relatively large growth cones. We prepared primary cultures of bag cells according to the method of Kaczmarek and Strumwasser (5). The cultured cells formed growth cones shaped like a thin lamellipodium for 1 to 3 days.

Figure 1A shows a Pol-Scope image of a growth cone in which several different birefringent components can be distinguished. Very prominent are radially aligned fibers that have the same location as the previously reported actin bundles (1, 6). In addition to the radially aligned fibers, Figure 1A shows

the high birefringence of the central region of the nerve process, which is filled with aligned microtubules and vesicles. At the leading edge of the growth cone, the cell membrane is imaged as a birefringent double layer. This must be interpreted with caution because the detailed structure of the double layer contains edge birefringence caused by the difference between refractive indices of the cytosol and of the extracellular medium (7, 8).

To confirm whether the radially aligned birefringent fibers are actin-based, we examined the effect of externally applied cytochalasin B. Cytochalasin B is well known to prevent polymerization of actin filaments in growth cones of the *Aplysia* bag cell neuron (1). When we added cytochalasin B to the external medium at the final concentration of 10 μ M, all the radially aligned fibers were eliminated within 1–2 min (Fig. 1B). The radially aligned fibers reappeared several minutes after removing cytochalasin B from the extracellular medium. The effect of cytochalasin B on the birefringent fibers is similar to the effect on actin bundles reported by Forscher and S. J. Smith (1). Moreover, in fixed non-cytochalasin-treated cells, the radially aligned fibers were fluorescently labeled with rhodamine-phalloidin, which selectively binds to actin filaments (Fig. 1C). These results suggest that the radial bundles are indeed actin-based.

The filamentous actin network in a neuronal growth cone shows a constant retrograde flow in DIC images (1). The retrograde flow in growth cones was also recognized in time lapse movies of Pol-Scope images. Moreover, we found that radially aligned actin bundles showed lateral associations as well as growth and retraction patterns.

Both the Pol-Scope and DIC can reveal molecular architecture directly in living cells. Pol-Scope images of growth cones (Fig. 1A, B, D) delineate birefringent objects against a dark non-birefringent background. Most importantly, image contrast of birefringent objects is independent of their orientation in the

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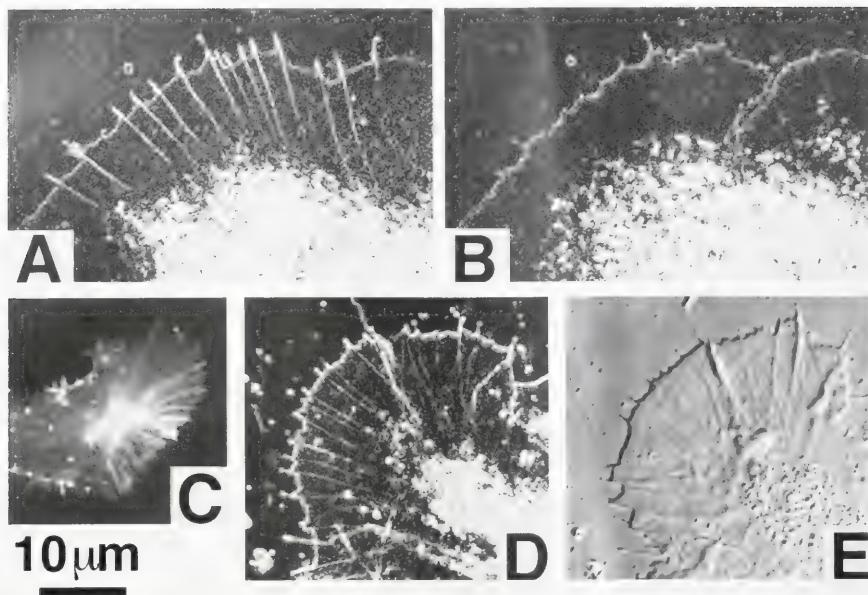


Figure 1. *Aplysia* neuronal growth cone. A,B: effect of cytochalasin B on the growth cone. (A) Pol-Scope image of the growth cone before application of cytochalasin B. (B) after application of cytochalasin B. C: Rhodamine-phalloidin stain of the growth cone demonstrating F-actin. D, E: Comparison of Pol-Scope image with DIC image. (D) Pol-Scope image. (E) DIC image. Pol-Scope images show the measured retardance magnitude in shades of gray (black is zero, white 1 nm retardance).

field of view. This is a significant difference between the Pol-Scope and the traditional polarized-light microscope. These qualities achieved through optical and computational image processing give the Pol-Scope an advantage compared with DIC (See Fig. 1D, E), in revealing molecular fine structure such as thin actin bundles. Furthermore, Pol-Scope images can be interpreted quantitatively because they are measurements of object retardance at each image point. On the other hand, DIC achieves image contrast through optical processing alone, allowing one to see a live image through the eye piece. Also, DIC more sensitively can detect spatial changes in refractive index or in optical path length. Therefore, both methods are often complementary and have advantages in specific applications.

In this report, we have demonstrated that the Pol-Scope can reveal actin-based structures in the growth cone. We are currently analyzing the quantitative results provided by the Pol-Scope with regard to the actin dynamics in living growth cones.

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Inhibition of Microtubule-Dependent, Minus-End Directed Transport of Axoplasmic Organelles by an Antibody Specific for the Intermediate Chain of Dynein

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Cytoplasmic dynein is a multi-subunit complex consisting of two heavy chains (HC) and several intermediate (IC) and light chains (LC). The intermediate chain of cytoplasmic dynein has been postulated to be responsible for docking the motor complex to membranous organelles (1, 2). The goal of the present study was to determine the role of the dynein IC in retrograde movement of squid axoplasmic organelles. Cytoplasmic dynein is responsible for retrograde (minus-end directed) transport (3, 4). We used an antibody (m74-2) specific for the IC of dynein (2) to block the function of dynein intermediate chain.

To test the specificity of this antibody, axoplasm of squid giant axons was extruded into axoplasmic buffer dilute 1:4 (4 mM Hepes, 2 mM MgCl₂, 0.5 mM CaCl₂, 1.5 mM EGTA, 100 mM K-aspartate, 18.5 mM taurine, 10 mM betaine, 7.5 mM glycine, pH 7.2) (5), then analyzed by SDS-PAGE and immunoblot. The antibody m74-2 recognized dynein IC in prepara-

tions of whole axoplasm, as well as membranous organelles isolated by flotation in a sucrose step gradient (Fig. 1A).

DIC video-microscopy of control axoplasm preparations in 1/4 strength axoplasmic buffer containing 2 mM ATP revealed vesicle motility, in both directions, within the bulk of the axoplasm, as well as along single microtubules at the periphery of the bulk. Treatment of axoplasm with the monoclonal antibody m74-2 caused no significant change in minus-end (retrograde) directed transport inside the bulk of extruded axoplasm. Treatment with 10 μ M vanadate, an inhibitor of cytoplasmic dynein, also failed to inhibit retrograde movement significantly. The lack of a complete inhibition of retrograde transport within the bulk of the axoplasm could be explained by the presence of actin-dependent motility (5). In contrast, only uni-directional movement was observed along single microtubules at the edge of the bulk in the presence of m74-2. Because the polarity of microtubules in the axoplasmic preparation was unknown, we therefore decided to study the effect of the antibody on organelle transport on microtubules of known polarity.

Axonemes of sea urchin sperm (kindly provided by Phong Tran, 6) were used as seeds to obtain polarized microtubules

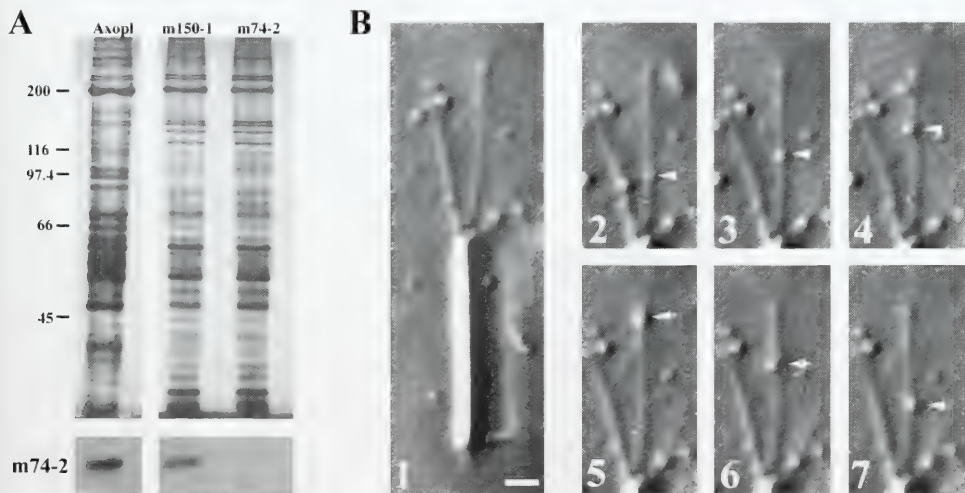


Figure 1. Inhibition of cytoplasmic dynein in squid axoplasm. (A) Silver-stained SDS-PAGE and m74-2-stained immunoblot of extruded axoplasm (Axopl), isolated membranes treated with control antibody (m150-1), and isolated membranes of axoplasm treated with m74-2 (m74-2). (B) DIC video-microscopy of organelles moving along microtubules assembled onto fragments of axonemes (1); (2–4), plus-end directed movement; (5–7), minus-end directed movement at a 1-s interval. Bar = 1 μ m.

(Fig. 1B). Fragments of axonemes were absorbed onto cover glasses by perfusion through a 2–3 μ l microscope chamber. The chamber was then perfused with 1 mg/ml purified bovine brain tubulin in buffer (100 mM K-Pipes, 2 mM $MgCl_2$, 2 mM EGTA containing 10 μ g/ml taxol, 1 mM GTP). After 30 min of incubation at room temperature, microtubules had assembled preferentially at the plus-end of the axonemes. To obtain a membrane fraction, we homogenized axoplasm in motility buffer containing 50 mM nocodazole, to depolymerize endogenous microtubules, and 5 μ M cytochalasin D, to depolymerize endogenous actin filaments; the homogenate was centrifuged at $10,000 \times g$ for 5 min. The membrane fraction, either with the addition of 0.5 mg/ml control antibody or 0.5 mg/ml dynein-IC specific antibody (m74-2), was perfused through the chamber, and vesicle movement along microtubules assembled onto the plus-end of axonemes was monitored by DIC video-microscopy.

In the presence of control antibody, we observed plus- and minus-end directed movement (Fig. 1B). The frequency of plus-end directed movement was 0.37 ± 0.11 events per μ m length of microtubule per minute ($n = 52$ events, 28.4 μ m, 15 min) and that of minus-end directed movement occurred at 0.16 ± 0.06 events/ μ m/min ($n = 24$ events). In the presence of antibody m74-2, only plus-end directed movement was observed (0.38 ± 0.04 events/ μ m/min), so the frequency of plus-end directed movement was not altered. The absence of minus-end directed movements in the presence of the dynein IC specific antibody demonstrated the importance of dynein IC for the function of the dynein motor complex.

To gain a better insight into the molecular mechanism of the inhibition caused by the antibody, we incubated homogenized axoplasmic extracts with control antibody or dynein IC specific antibody and analyzed the isolated vesicle fractions by immunoblot. While dynein IC was present in vesicle fractions treated with control antibody, no dynein IC could be detected in membrane fractions treated with m74-2 (Fig. 1A).

These results demonstrate that the monoclonal antibody m74-2 blocks dynein-driven organelle movement by dissociating the dynein complex from the membrane surface. We therefore conclude that dynein IC plays an essential role in the dynein motor complex and serves to link dynein to membranous axoplasmic organelles.

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Characterization of Antibodies to the Head and Tail Domains of Squid Brain Myosin V

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A rabbit polyclonal antibody made to purified squid myosin V (1) was used to show that this myosin motor is localized to ER vesicles isolated from extruded axoplasm (2). To understand more clearly the properties of this organelle motor, we have begun to clone and sequence the gene for squid myosin V. Proteolytic fragments of myosin V purified from squid optic lobes were partially sequenced (Howard Jaffe, NIH, Bethesda, MD), and the sequence data were used to design degenerate primers for PCR. Two clones, a 650 base pair fragment in the head domain of the protein and a 500 base pair fragment in the tail domain, were sequenced. The two cloned sequences of squid brain myosin were compared with full-length sequences of the other members of the myosin V class. The squid brain myosin sequences showed high homology to p190 (chick), myr6 (rat), and dilute (mouse) myosins V. A peptide of 14 amino acids (LASNPIMESFGNAK) in the head domain of squid myosin V, near the ATP binding site (Fig. 1A), was synthesized and used

for antibody production (HDP/LASN) in rabbits. In addition, a peptide of 16 amino acids (QLLQPRKTDADVDSVC) from the tail domain was synthesized and used for antibody production (TDP/QLLQ).

The HDP/LASN and TDP/QLLQ antibodies were affinity purified on a Thiol coupling gel column (QCB Inc., Hopkinton, MA), and the affinity purified antibodies were used to probe blots of squid brain myosin V and axoplasm. On western blots, the HDP/LASN antibody gave a strong reaction to purified squid brain myosin V (p196, Fig. 1B, lane 1) but did not react with the p196 band in axoplasm (Fig. 1B, lane 2). The lack of reactivity with axoplasm is probably due to the small amount of p196 in whole axoplasm and the lower affinity of this antibody for squid myosin V compared to the tail domain antibody. This antibody reacted with an unidentified contaminating protein at 55 kDa in the squid brain myosin V fraction (Fig. 1B, lane 1), but this protein was not present in axoplasm (Fig. 1B,

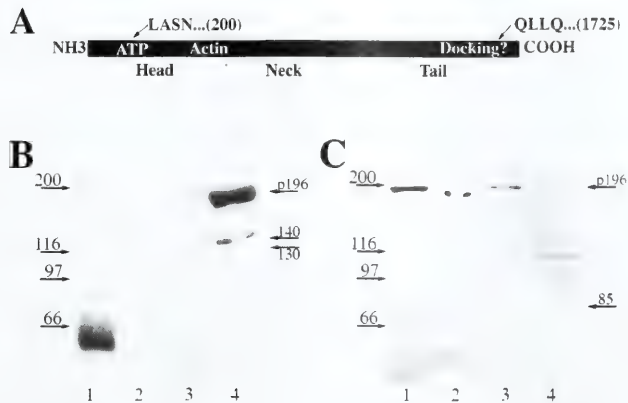


Figure 1. (A) Diagram of the amino acid sequence of squid brain myosin V, illustrating the position of the two peptides (LASN . . . and QLLQ . . .) used to generate antibodies. The ATP and actin binding sites in the head domain, and the putative membrane docking site in the tail domain, are indicated. The numbers 200 and 1725 indicate the number of residues from the N-terminal for the two squid peptides relative to the chick myosin V sequence. (B) Blot probed with the HDP/LASN, the head domain antibody. Lane 1, purified squid brain myosin V; lane 2, axoplasm from the squid giant axon; lane 3, squid brain myosin V calpain digest; lane 4, chick brain myosin V. Molecular weight markers are indicated to the left of the blots. (C) Blot probed with the TDP/QLLQ, the tail domain antibody. Lane 1, purified squid brain myosin V; lane 2, axoplasm from the squid giant axon; lane 3, squid brain myosin V calpain digest; lane 4, chick brain myosin V.

lane 2). The head domain antibody also gave a very strong reaction to chick brain myosin V (Fig. 1B, lane 4), purified according to the procedure of Cheney *et al.* (3). The TDP/QLLQ tail domain antibody reacted strongly with purified squid brain myosin V (Fig. 1C, lane 1), and the equivalent band in axoplasm (Fig. 1B, lane 2). Chick brain myosin V was not recognized by the antibody (Fig. 1C, lane 4). Therefore, both antibodies exhibited high titer and high specificity for squid myosin V.

The calcium-dependent protease calpain cleaves chick brain myosin V into two stable peptide fragments of 130 and 80 kDa, corresponding to regions of the head and tail domains respectively (4). With further incubation, the 130 kDa fragment is cleaved into smaller fragments. Calpain cleavage of myosin has been hypothesized to play a physiological role in neurons. To test the effect of calpain on squid brain myosin V, a mixture containing 1:4 calpain to myosin (w:w) was incubated for one hour, as described for chick myosin V (5). After calpain digestion of squid brain myosin V, the head domain antibody, HDP/LASN, recognized a pair of bands at 130 and 140 kDa (Fig. 1B, lane 3); and the tail domain antibody TDP/QLLQ, recognized an 85 kDa band (Fig. 1C, lane 3). Therefore, calpain effectively cleaved purified squid brain myosin V into fragments with molecular weights similar to those generated from chick brain myosin V. These results suggest that the calpain cleavage sites are in similar locations in the two proteins. In chick brain myosin V, the calpain cleavage site was one amino acid away from the PEST motif (5), a sequence that has been hypothesized to

be associated with cleavage by calpain (6). These results lead us to hypothesize that the PEST motif in squid myosin V is located about the same distance away from the carboxyl terminal in the squid myosin V as in chick myosin V.

In summary, we have successfully produced two antibodies to peptides, one located in the head domain near the site of ATP hydrolysis, and the second located in the tail domain in the predicted membrane docking site. Each antibody reacted with the purified protein and recognized a unique calpain cleavage fragment. We will use these antibodies to show that myosin V is the specific motor for the transport of vesicles, including the transport of ER vesicles on actin filaments.

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Dynamics of GFP-coronin and Eupodia in Live *Dictyostelium* Observed With Real-time Confocal Optics

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Temporally and spatially specific interactions between actin and its binding proteins are thought to play crucial roles in cell motility. Coronin, a 55-kD protein whose genetic sequence has a similarity to G-protein β -subunits, binds to actin in a Ca^{2+} -insensitive manner. This protein has been localized in a crown-shaped cortical structure in *Dictyostelium* amoebae (1). Gene knockout of coronin in amoebae causes severe defects in cytokinesis and cell motility (2); phagocytosis (3); and fluid-phase

macropinocytosis (4). Accumulation of coronin at the leading edge of chemotactically activated amoebae has been demonstrated with a green fluorescent protein (GFP)-coronin fusion protein (5).

In the current study, we have analyzed the dynamic integration of GFP-coronin into the actin cytoskeleton, in particular the eupodia, to probe the architectural dynamics of actin in live *Dictyostelium* amoebae. Amoebae prepared by the agar-overlay method form actin-rich anchorage structures, or "eupodia" (true feet), that contain actin-binding proteins, such as myosin-IB and α -actinin (6), as well as coronin and ABP-120 (Fukui, unpub.). As we document here, coronin, presumably bound to actin, appears in exceptionally high concentration each time a eupodium is transiently formed at the base of an extending

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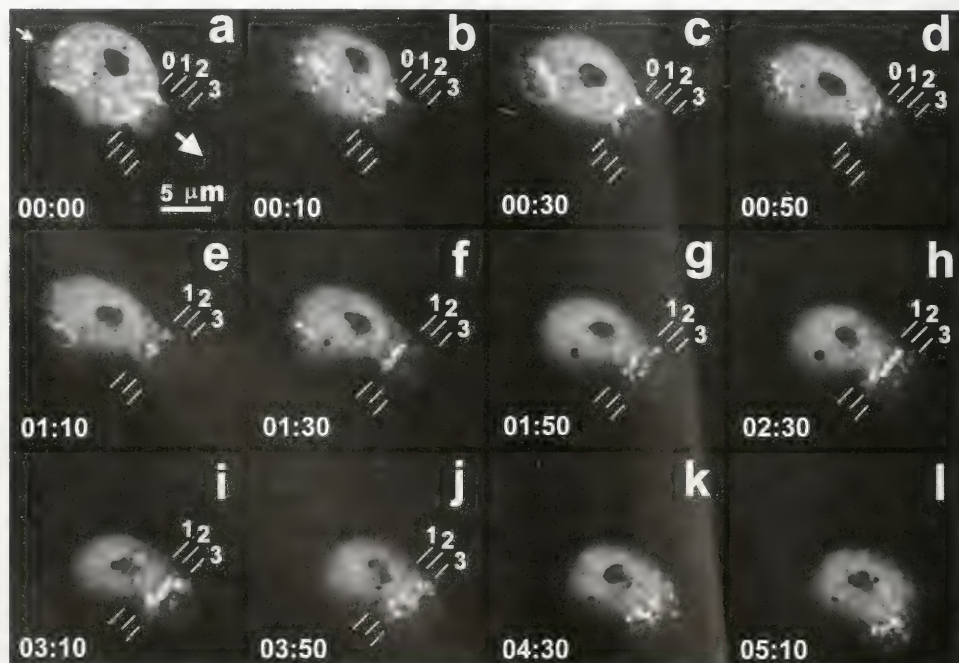


Figure 1. Selected frames showing the dynamic assembly of GFP-coronin into developing eupodia (bright fluorescent dots). The amoeba is initially retracting a pseudopodium (panel a, small white arrow), and its eupodia disappear in less than 2 min (a–g). Following the initiation of a new array of eupodia (line 0), the amoeba started projecting a new pseudopodium (a: large white arrow). As it continued to extend this pseudopodium over the next 5 min, the amoeba progressively generated and abolished new rows of eupodia (lines 1–3). Time: min:s.

pseudopodium. Because eupodia are small ($\sim 1 \mu\text{m}$ in diameter), dynamic (average life is less than 3 min), and are located at the amoeba-agarose interface, we observed the agar-overlaid amoebae with a real-time confocal system (7) to achieve high temporal and spatial resolution. The amoebae express either an S65T-GFP-coronin fusion protein in wild type Ax2, or a wild type GFP-coronin in the null coronin mutant. S65T-GFP-coronin was integrated into a vector, whereas GFP-coronin was integrated into chromosomes by gene replacement.

We found that *Dictyostelium* amoebae expressing GFP-coronin are very sensitive to the 488-nm blue laser beam used for confocal fluorescence imaging; they rounded up within a few minutes of continuous or shorter than 5 seconds' observation. To minimize this effect, we first located the amoeba by non-confocal epifluorescence, using reduced intensity blue excitation, for 10 to 20 seconds, on a Nikon E800 microscope equipped with a $60\times/1.4 \text{ N.A.}$, or $100\times/1.4 \text{ N.A.}$, plan apo oil immersion objective lens. As quickly as possible, we focused on a cell, closed the epifluorescence shutter, and switched out the epifluorescence dichromatic mirror. We then opened the electric shutter in the real-time, spinning-disk confocal scanning unit (Yokogawa CSU-10) attached to the microscope. The video-rate confocal image was integrated on-chip for 6 to 15 video frames (0.2–0.5 s) in a Hamamatsu Chilled CCD camera (C-5985). Synchronous opening of the electric shutter, and time-lapse acquisition (at an interval of 10 s) of the image, were controlled with an image processing computer (MetaMorph; Universal Imaging Corp.). All images were saved as tiff files into the hard drive and processed for the production of video movies and image panels using MetaMorph and Photoshop (Adobe).

High-resolution confocal video images of motile *Dictyostelium* amoeba showing the dynamic accumulation of GFP-coronin, as well as its absence in several organelles (nucleus, mitochondria, contractile vacuoles) were shown at the 1997 MBL General Scientific Meetings. Our observations demonstrate that GFP-coronin is dynamically integrated into the actin cytoskeleton in the pseudopodia, especially the eupodia, and apparently

in endocytotic cortical structures. The brightly fluorescent eupodia do not move relative to the substratum. Instead, as shown in Figure 1, when a new row of eupodia appears, the previous row disappears in a well-coordinated manner. Both the assembly and disassembly of GFP-coronin into a single eupodium takes place in less than 30 s. It is unlikely that the observed dynamics of GFP-coronin in eupodia was caused by exposure of the amoeba to 488 nm light because identical pattern of eupodia dynamics can be recognized without fluorescence excitation by their high refraction index (6).

Since coronin binds to F-actin (1); the eupodia contain a high concentration of F-actin (6); and injected rhodamine-actin pattern is similar to that of GFP-coronin (Yumura and Fukui, in prep.), the intense fluorescence of GFP-coronin in the eupodia is likely to reflect the high concentration of F-actin filaments that appear in these transient organelles. However, detailed interpretation must await clarification of the physiological role of coronin and the physico-chemical conditions that regulate the binding of coronin to actin.

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Photodynamic Effect of 488-nm Light on Eosin-B-stained *Spisula* Sperm

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We describe here striking, sudden color changes induced photodynamically in Eosin-B-stained *Spisula* sperm observed by real-time confocal microscopy. Motile sperm of *Spisula solidissima* in a perfusion chamber were observed with a real-time,

spinning-disk confocal scanning unit (Yokogawa CSU-10) attached to a Nikon E-800 microscope equipped with a $100\times/1.4 \text{ NA}$ Plan Apo oil immersion lens. The sperm were suspended in a solution containing 0.1% Eosin B [a dye earlier reported to stain only dead sperm (1)] in seawater. To check for motility and to observe the sperm as they attached to the coverslip, the sperm were first observed on or near the surface of the coverslip of the perfusion chamber with trans-illuminated white light. Then the shutter in the confocal scanning unit was

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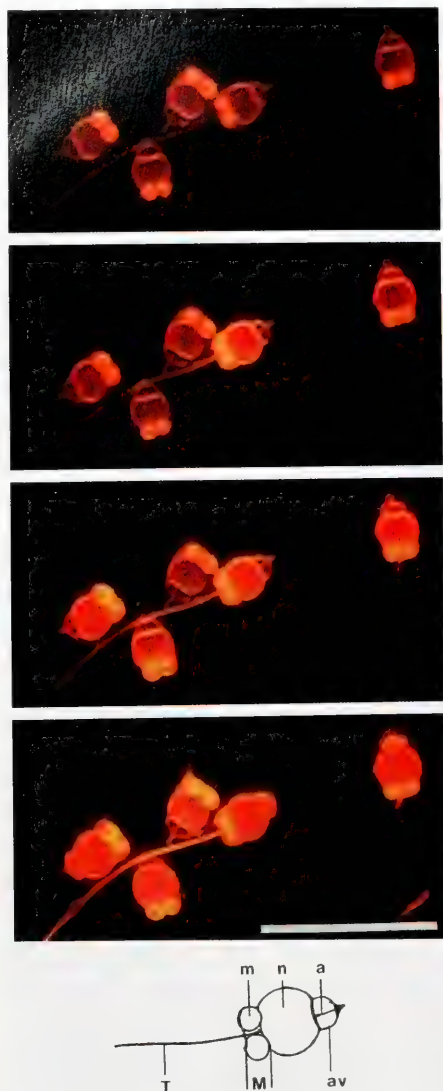


Figure 1. Selected frames from confocal video sequence showing the striking photodynamic changes in fluorescence and morphology of Eosin-B-stained *Spisula* sperm exposed to the 488-nm (fluorescence-exciting) laser beam. Top to bottom panels: 13, 14, 15, and 31 s after exposure to 488-nm laser beam, sperm are labeled a-e from left to right. Except for sperm d, much of the tail is invisible owing to the shallow field depth of the confocal optics. Scale bar = 10 μ m. Bottom: Schematic diagram of *Spisula* sperm. a: acrosome. av: acrosomal vesicle. M: midpiece. m: mitochondrion. n: nucleus. T: sperm tail (part only shown).

opened to produce the fluorescent confocal image excited by the 488-nm beam from an Argon ion laser. The weak fluorescence images of the sperm were sequentially integrated on-chip for 20 frames (0.66 s) on a chilled color CCD camera (Dage-MTI Model 330) and observed continuously. The RGB signal from the camera was displayed on a color video monitor, as well as recorded to an S-VHS video tape recorder, after converting to Y/C format (Truevision Vid I/O) and adjusting the color balance and background signal level with an analog video processor (Elite Video BVP-4 Plus).

Immediately upon exposure to 488-nm excitation, four mitochondria, owing to their faint but distinct yellow fluorescence, could be seen in the mid-piece by through-focusing. The membranes outlining the acorn-shaped sperm head, acrosomal vesicle, short acrosomal filament within the vesicle, and the sperm tail also showed very weak yellowish-orange fluorescence (Fig. 1, top panel and schematic). After about a 15-s exposure to the 488-nm laser beam (0.01 mW per μ m² at the specimen), the same beam that also provided the confocal fluorescence excitation, the sperm stopped moving and showed a dramatic sequential change in fluorescence. First, the four spherical mitochondria suddenly (<1 s) acquired a much brighter yellow fluorescence and became somewhat larger (second panel sperm d; third panel a, b, e). Within another second, the nucleus began acquiring a red fluorescence that often started from a zone adjacent to the mid-piece and propagated through the nucleus at a velocity of about 0.5 μ m/s (second panel d; third panel b, e). In the meantime, the nucleus had become quite swollen and acquired a uniformly strong, bright-red fluorescence. About 10 s after the nucleus had acquired a uniform fluorescence, the acrosomal cup suddenly lit up with a pinkish-yellow fluorescence (fourth panel a, d, e). In the meantime, the acrosomal process and the sperm tail had gradually acquired a yellowish-orange fluorescence which became stronger with time over the next several minutes (top to bottom panels).

Control sperm exposed to the 488-nm laser beam, but not stained with Eosin B, continued to swim for at least many minutes and did not show the shape changes characteristic of those that had been stained.

Taken together, the observed sequential changes in fluorescence and morphology show that the photodynamic change initially takes place at or near the mid-piece membrane and then appears to spread to the different cellular compartments. Most interestingly, the time delay before entrance of the dye into new compartments, the color of fluorescence, and its rate of propagated change within each compartment, all showed distinct differences depending on the compartment within the minuscule sperm head and tail.

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Effect of Gossypol on *Spisula* Sperm Observed with Real-time Confocal Microscopy, Polarized Light Microscopy, and Video Microscopy

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Ingestion or injection of gossypol suppresses sperm production and sperm motility *in vivo* (reviewed in ref. 1). We report here an *in vitro* assay for observing gossypol action on individual sperm. A sample of the sperm suspension was added to a perfusion chamber consisting of a polylysine-coated coverslip and a glass slide separated by two strips of double-sticky tape. Sperm from the bivalve mollusc, *Spisula solidissima*, were suspended in a solution of filtered seawater and 0.1% Eosin-B. Eosin-B stains the nucleus of only dead sperm red (2), and it therefore serves as a marker for dead sperm. The sperm heads adhered tightly to the coverslip, often leaving the flagella free to move in solution. Filtered seawater containing 0–100 μM gossypol was perfused through the chamber. Observations were performed either with a real-time confocal microscope (3), the

Pol-Scope polarized light microscope (4), or a bright field video microscope.

Each of the above microscopy techniques allow for observation of different aspects of sperm morphology, motility, and vitality. The real-time confocal, by imaging the fluorescence of Eosin-B-stained sperm, allows comparison of morphological changes between control and gossypol-treated sperm. The Pol-Scope allows observation of cellular fine-structures. Finally, bright field video microscopy has high temporal resolution (30 frames/s video rate) for quantifying rates of action of gossypol on sperm.

The effects of gossypol on *Spisula* sperm occurs in the following sequence: (1) cessation of flagellar motility; (2) abrupt swelling of the midpiece region where the mitochondria reside; and (3) swelling of the sperm head (Fig. 1a'–c'). The effects of gossypol, scored by Eosin-B staining, were concentration dependent (Fig. 1d). For instance, at the lowest gossypol concentration used (10 μM), 100% of the sperm died within 90 min, whereas at the highest gossypol concentration used (100 μM), 100% of the sperm died within 2.5 min. The rate of

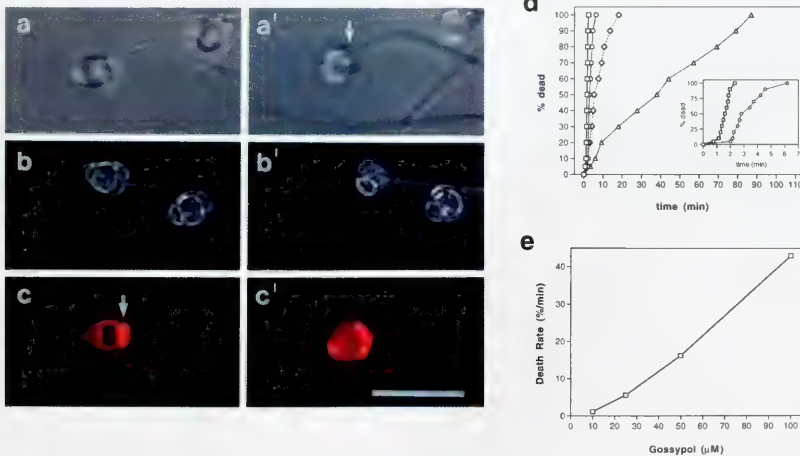


Figure 1. Before (a, b, c) and after 100 μM gossypol treatment (a', b', c') of *Spisula* sperm adhering to poly-lysine coated coverslips: (a') bright field microscopy showing prominent swelling of the midpiece (arrow) of sperm after gossypol treatment; (b') polarized light microscopy showing rearrangement of cellular fine-structures as well as swelling of sperm membrane after gossypol treatment; (c') confocal microscopy showing morphological defect and displacement of mitochondria (arrow in panel c) in the midpiece region after gossypol treatment. (d) plot of percent of total sperm dying vs. time at 10 μM (triangle), 25 μM (diamond), 50 μM (circle), and 100 μM (square) gossypol. (e) plot of rate of sperm death vs. gossypol concentration. Scale bar 5 μm .

sperm death was approximately linear at each concentration of gossypol used (Fig. 1d), and the rates of death increased with increasing gossypol concentration. For example, death rate is 1%/min at 10 μ M gossypol, and 40%/min at 100 μ M gossypol (Fig. 1e). Control sperm in the absence of gossypol remained alive and motile for more than 2 h.

It is not clear at the present what the molecular mechanism is of gossypol action on sperm. However, our light microscopic study is consistent with the hypothesis that gossypol directly perturbs the mitochondria, creating an imbalance in the mitochondrial transmembrane potential, and inducing osmotic swelling.

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Fellowship to PTT; and the F. R. Lillie Fellowship to MHB; and Dage-MTI, Hamamatsu Photonics, Nikon, Olympus Optical, Yokogawa Electric, Universal Imaging, and Carl Zeiss for research support and loan of equipments to SI. RO is supported by NIH grant GM49210.

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Effect of Gossypol on the Ultrastructure of *Spisula* Sperm

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Gossypol is a phenolic dialdehyde derived from the cotton plant (*Gossypium herbaceum*). It decreases sperm motility and is being studied as a potential male contraceptive (1).

This report describes the action of gossypol acetate extracted from *Gossypium herbaceum* grown in Brazil on structural aspects of *Spisula* sperm, as analyzed by scanning-electron-microscopy (SEM), transmission-electron-microscopy (TEM), and freeze-fracture.

Control sperm and sperm exposed to 50 μ M gossypol for 10 to 30 min were fixed in 2.5% glutaraldehyde in filtered seawater and processed for SEM, TEM, and freeze-fracture according to conventional methodology (2).

SEM reveals that after 10 min exposure to 50 μ M gossypol, *Spisula* sperm undergo a pronounced swelling of the midpiece accompanied by an irregular appearance of the cell membrane surrounding both the midpiece and sperm head (Fig. 1B). Control sperm have a smooth cell surface and a mitochondrial midpiece of normal size (Fig. 1A).

When ultrathin sections of similarly treated sperm cells (10 min exposure to 50 μ M gossypol) are studied, the midpiece can be observed to be swollen (Fig. 1D) compared to control sperm (Fig. 1C). The treated sperm have small cytoplasmic granules among the mitochondria, which appear normal. These granules

are absent in control sperm. With longer exposure to gossypol (50 μ M) for up to 30 min, the accumulation of granules increases and swelling of the midpiece continues.

Freeze-fracture replicas of sperm exposed to 50 μ M gossypol for 10 min show swelling of the midpiece and reveal that the internal layer of the cell membrane has an altered distribution of particles, compared to control sperm. In the cell membrane of non-treated sperm, particles in the internal layer are distributed randomly (Fig. 1E). In the membrane of treated cells, particles are completely absent in some areas (Fig. 1F).

We conclude that exposure to gossypol alters the structure of the cell membrane of *Spisula* sperm, initially at the midpiece and subsequently around the sperm head. This morphological effect is closely related to changes in sperm motility, membrane permeability, and cell viability.

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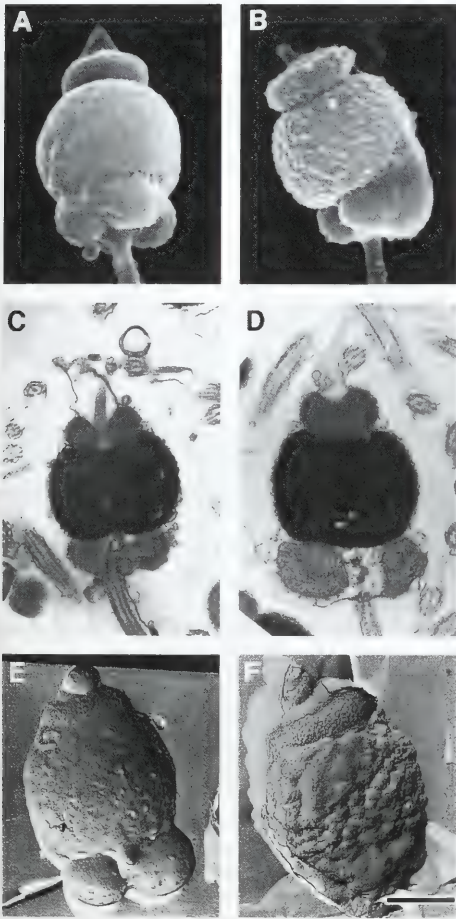


Figure 1. Electron micrographs of *Spisula* sperm; midpiece and head. Control sperm (A, C, E) and sperm exposed to 50 μ M gossypol for 10 min (B, D, F). SEM shows the change in the head surface and swelling of the midpiece (B) as compared to the control (A). TEM reveals swelling of the midpiece with the appearance of many cytoplasmic particles in the gossypol treated sperm (D); control sperm appears normal (C). Freeze-fracture replicas also reveal changes in the cell membrane with altered distribution of intramembrane particles in the gossypol treated sperm (F) as compared to the control sperm (E). For identification of cellular components, see schematic in S. Inoue et al. (3). Scale bar = 1 μ m.

Reference: *Biol. Bull.* **193**: 229–230. (October, 1997)

Orientation of Motile Unicellular Algae to Oxygen: Oxytaxis in *Euglena*

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Oxygen sensing by a microorganism was first demonstrated by Engelmann (1) in his experiment that determined the photosynthetic action spectrum. In motile bacteria, a positive chemotactic response to oxygen was subsequently observed as a net migration to environments that are better aerated (2). In fungi, growth responses toward oxygen are thought to be responsible

for self avoidance in hyphae (3). In higher plants, oxygen has only recently been shown to play a direct role in determining root orientation (oxytropism) (4). To understand these responses and the associated pathways of signal transduction in higher plants, it may be easier to first study the response in an algal system.

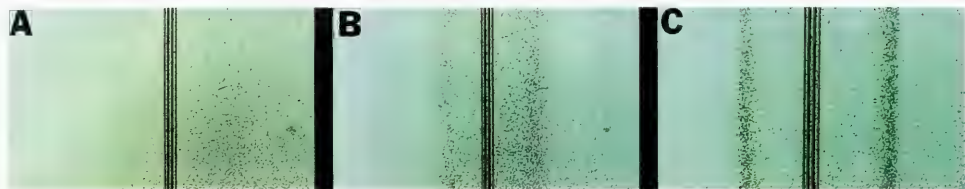


Figure 1. Time-series photographs of the oxytaxis response of *Euglena gracilis*. (A) Random distribution of cells before air flow is initiated in the silicone tube. (B) Distribution of cells after 10 minutes of exposure to the artificial oxygen gradient. (C) Steady-state distribution of *Euglena* shown after 20 minutes. Tube diameter = 150 μm .

To test whether a motile, unicellular, freshwater, photosynthetic alga (*Euglena gracilis*) can sense and respond to oxygen (oxytaxis), an airtight stainless steel cuvette with an optical path of 170 μm (Dormier, Friedrichshafen, Germany) was modified to include a system capable of producing and maintaining an oxygen gradient. The oxygen gradient was created by pumping air through a miniature silicone tube (150 μm outer diameter, 50 μm inner diameter) running through the center of the chamber. *Euglena gracilis*, strain Z, was inoculated in a complex growth medium (5) and grown for 12 days at 23°C under continuous lighting (2.5 W m^{-2}). To decrease the concentration of dissolved oxygen in the medium, the culture was exposed to the dark for 24 h before being transferred to the test chamber. The respiration of the cells decreased dissolved oxygen concentrations, and the dark treatment inhibited photosynthetic oxygen evolution. The cells were kept in the dark during the experiments. Reorientation of the cells in the test chamber was photographed at 10-min intervals with a 35-mm camera mounted on a dissecting microscope. All photographs were taken immediately after the light source was turned on. The experiment was replicated 5 times with three different cultures.

Euglena gracilis responded to the oxygen concentration gradient by swimming toward higher concentrations of O_2 (Fig. 1). At time zero (Fig. 1A) the air pump was turned on and an oxygen concentration gradient began to develop away from the tubing. Since this gradient was initially shallow, the cells that encountered it moved toward the surface of the tube (Fig. 1B). The gradient continued to move out into the medium, thereby attracting more cells to move toward the tube. As the gradient moved into the medium, $p\text{O}_2$ of the medium increased and the initial wave of cells moved with the flux of oxygen, away from the tube. This continued until the metabolic oxygen consumption of the cells within this artificial oxygen gradient matched the oxygen-delivering capacity of the tubing. At that point the gradient ceased to extend into the medium and a boundary characterized by a high cell density was formed parallel to the surface of the tubing (Fig. 1C). This steady-state cellular distribution pattern could subsequently be disrupted by either turning off the air flow through the tube or applying constant illumination. Turning off the air flow caused the gradient to

retreat back to the tube. Subsequently the cells moved back to the tube surface before becoming randomly distributed when the oxygen gradient collapsed. Illumination rapidly disrupted the distribution pattern as the cells began to photosynthesize in response to light and produce oxygen. Since these cells then had an internal source of oxygen, they were no longer able to sense any external oxygen gradient and responded by changing to a random distribution.

These results show that unicellular algae can sense and respond to oxygen in the dark when the photosynthetic apparatus is not active and oxygen is not being produced by the chloroplasts. Previous investigators have described red-light-induced accumulation of *Euglena* and hypothesized that this response was not a true phototaxis (6). The results of these experiments support this hypothesis. A photosynthetically active radiation point source could result in localized photosynthetic oxygen production, and a resulting oxygen gradient that could attract cells from non-illuminated regions of the culture.

It has been well documented that higher plants orient growth by sensing environmental clues such as gravity, water, and light. Recent work has shown that plants can also sense oxygen and respond tropically (4). These experiments with *Euglena* further demonstrate that this response to oxygen is not limited to higher plants, but is also present in unicellular motile algae. These results thus suggest that oxygen-sensing mechanisms are not recent evolutionary advancements associated with terrestrial plants, but may have been present in an algal protist ancestor.

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Consumption of Oxygen by Isolated Skate Retinal Photoreceptors

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A major problem in understanding the regulation of cellular metabolism is the heterogeneity of tissue structure, not only with regard to the cellular composition, but also within the individual cells. An ingenious step was the development of a method for measuring the oxygen consumption of a single isolated cell, though without spatial resolution around the cell itself (1). In this study, a solitary, amphibian rod photoreceptor was used. These cells come from the retina, a tissue with an especially high metabolic rate (2), reflecting primarily the contribution of the photoreceptors (3). Previous measurements of retinal oxygen consumption have suggested that the metabolic activity of the vertebrate retina varies with light intensity (4), but neuromodulators, such as dopamine, may also substantially alter the metabolic activity of retinal neurons (5, 6). With one exception (1), these studies have been conducted using whole retinas, composed of a variety of cell types. Consequently, it has been difficult to determine clearly the oxygen consumption of different categories of cell. Oxygen-consumption experiments performed on isolated, identifiable retinal cells, with good spatial resolution, would provide new information on the control and modulation of metabolic activity in photoreceptors and provide an approach applicable to numerous other varieties of cells.

The purpose of the present study was to measure oxygen consumption by isolated photoreceptors where there is a high degree of structural polarization, with improved spatial resolution. This involved the use of a new technique incorporating polarographic oxygen-selective microelectrodes in a self-referencing, non-invasive modality (7). In a polarographic oxygen recording, the electrical current needed to reduce molecular oxygen to H_2O is measured. The current is directly proportional to the concentration of oxygen in the solution. As with ion-selective electrodes, sensitivity is compromised by a random and unpredictable drift, the impact of which can be greatly reduced by using a self-referencing approach (7). Here the electrode is moved periodically between two positions near the cell membrane, and the differential current output is calculated as a function of local oxygen concentration and thus fluxes equivalent to the consumption of oxygen. The point of nearest approach is within $1\ \mu m$ of the membrane, the second position $10\ \mu m$ distant. The differential values are compared to a reference position tens of microns away. In all cases, the electrode is moved at a frequency of 0.3 Hz.

In the present experiments, oxygen-selective electrodes with final tip diameters of $1\text{--}4\ \mu m$ were prepared according to the

method of Linsenmeier and Yancy (8) giving an expected spatial resolution in the region of μm (2). The electrodes were polarized continuously at $-0.75\ V$ during recordings. The electrodes were calibrated in solutions containing nominally zero oxygen (prepared by bubbling with nitrogen for $\frac{1}{2}\ h$), 10% oxygen (prepared by bubbling with a 10% O_2 , 90% nitrogen

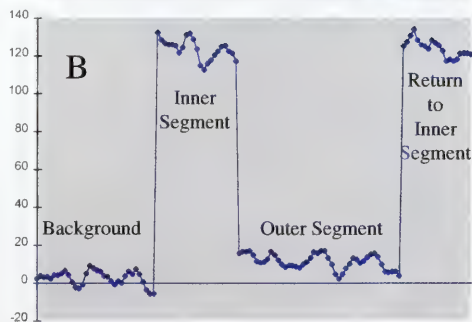
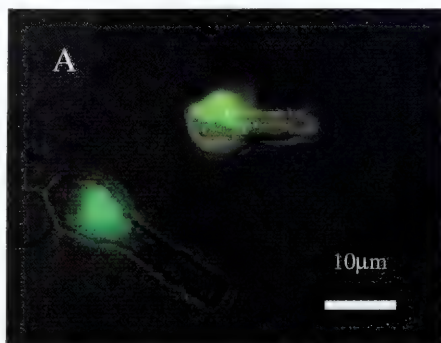


Figure 1. A. Mitochondria within isolated skate photoreceptors revealed by rhodamine fluorescence; staining can be seen in the ellipsoid area of the inner segment of the cells. Rhodamine ($75\ nM$) was added to the culture dishes containing the cells, left for 1 h, and then rinsed away with skate Ringer. The cells were stimulated with $545\ nm$ light and viewed with a barrier filter passing wavelengths greater than $590\ nm$. B. Oxygen consumption recording from a single photoreceptor. The electrode was placed first at a location $60\ \mu m$ distant from the cell, then within $1\ \mu m$ of the ellipsoid of the inner segment, then moved to the tip of the outer segment, and finally back once again to the inner segment. By convention, lower oxygen values near the cell are plotted as positive values. Axes: y axis, fA; x axis, 0–5 min.

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for $1/2$ h), and air (21%). The response of the electrodes, as a function of oxygen concentration, was linear.

Isolated photoreceptors from the all-rod retina of the skate (*Raja erinacea* or *R. ocellata*) were prepared according to an enzymatic dissociation protocol described in detail in Malchow *et al.* (9). The cells used in the present experiments possessed outer and inner segments but no obvious synaptic terminals. All of the recordings reported here were from light-adapted photoreceptors continuously bathed in bright white light.

Vertebrate photoreceptors are highly polarized in structure, with most of the mitochondria located in a specific portion of the inner segment called the ellipsoid. The rest of the cell is largely devoid of mitochondria (see Fig. 1A). This distinctly compartmentalized distribution should result in oxygen concentrations being lowest at the ellipsoid; *i.e.*, the oxygen consumption is high in this area, but very low near the tip of the outer segment. Indeed, when electrodes are situated next to the ellipsoid, we obtain differential readings indicative of a higher rate of oxygen consumption in that region (Fig. 1B). In eight cells examined, the average differential current measured next to the cell is $138.8 \text{ fA} \pm 41 \text{ fA}$. The conversion of these currents to an oxygen flux can be calculated by the formula:

$$\text{Flux } (\mu\text{mol cm}^{-2} \text{ s}^{-1}) = -D[(\Delta A \cdot S) \Delta r^{-1}]$$

where D is the diffusion constant for oxygen ($2.5 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$), ΔA (picoAmps) is the recorded current differential, S is the slope of the electrode ($\mu\text{mol ml}^{-1} \text{ picoAmps}^{-1}$), and Δr the distance between the measured points in cm. Applying this equation to our data, we find that the average amount of oxygen flux into the ellipsoid is $0.015 \pm 0.004 \mu\text{mol cm}^{-2} \text{ s}^{-1}$. Current readings obtained at the tip of the outer segment for the same eight cells are indistinguishable from readings at background, indicating no detectable oxygen uptake by the cells in this region.

Our data demonstrate that polarographic, self-referencing,

oxygen-selective electrodes can be used to monitor oxygen consumption from isolated retinal photoreceptors with a spatial resolution that reveals single cell inhomogeneities in the consumption of oxygen. We have shown significant differences in the oxygen concentration profile around cells, probably reflecting similar profiles within. We anticipate that these differences will have important physiological implications.

We thank Richard H. Sanger (MBL) for technical and programming support, Kasia Hammar (MBL) for assistance with cell culture and general laboratory support, Marshall Porterfield (MBL) for help with the oxygen flux calculations, and Robert Linsenmeier and Lissa Padnick (both of Northwestern University) for generously providing instruction in the preparation and use of the oxygen electrodes and for the gift of gold-plating solution. This work was supported by grants P41 RR01395 from the National Center for Research Resources to PJSS and EY09411 from the National Eye Institute to RPM.

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Comparison of Three Techniques for Evaluating Seasonal Gametogenesis in *Spisula solidissima*

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Spisula solidissima, the Atlantic surf clam, is widely used in reproductive research. This species is usually considered to produce ripe gametes once during the year in May to June (1, 2, 3), but dual cycles of gametogenesis have been reported in one location (4). Personnel at the Marine Resources Center (MRC) of the Marine Biological Laboratory have noted that

clams at different sites contain mature gonads at different times of the summer. Inability to predict the gonadal maturity of animals being collected results in wasteful harvest of unripe and partially ripe clams that cannot be used for research.

Descriptions of the gross reproductive morphology of the gonad stages is lacking. However, through histologic examination of gonadal tissue sections, five stages have been identified in the reproductive cycle of both sexes of *Spisula*: early active, late active, ripe, partially spawned, and spent (4). But histologic evaluation of gonadal tissue is time consuming, expensive, and requires sacrifice of the clam. In an effort to identify a quicker

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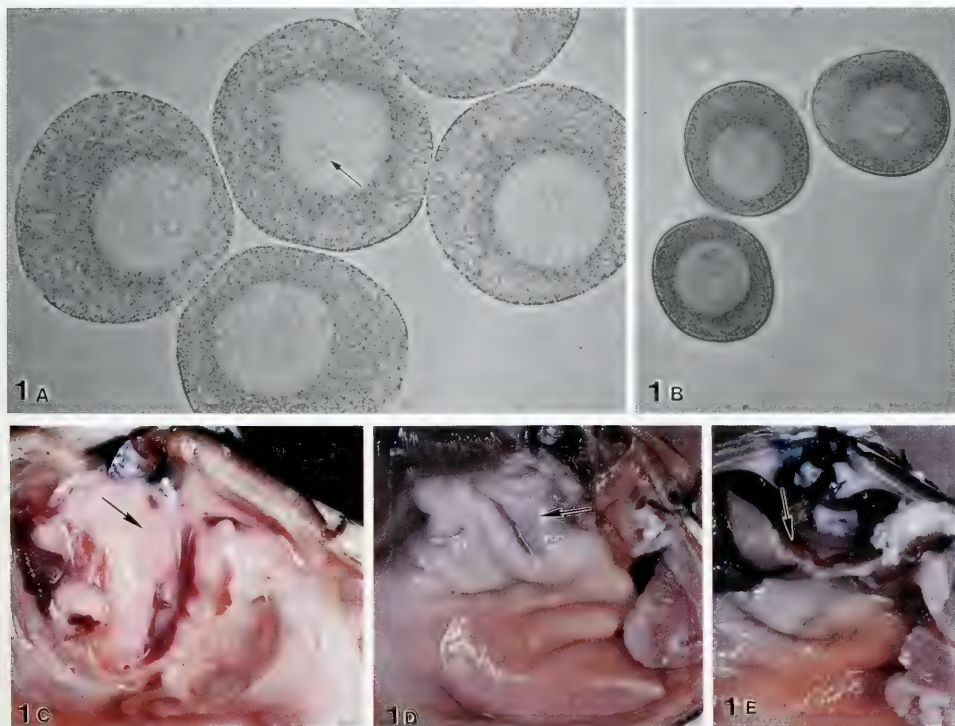


Figure 1. Samples of needle biopsy (A, B) and gross morphology (C-E) of *Spisula solidissima* gonad: Wet preparation of needle biopsies shows large eggs with well-developed amphinucleoli (arrow) from a ripe female (A) and small eggs from a female that is partially ripe (B). Photographs of gross morphology show a ripe female with abundant pink gonadal tissue and gonadal exudate (arrow) (C) and a spent clam with a collapsed body surface (arrow) (D) and an absence of gonadal tissue (arrow) (E).

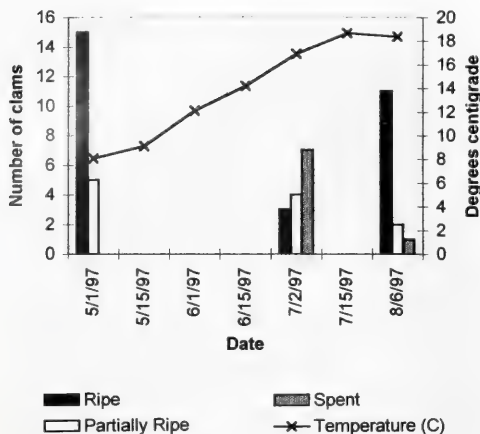


Figure 2. Number of surf clams examined from Menemsha Bay (1997) in each stage of gonadal activity at each collection time. Water temperatures at sediment level for dates identified are also plotted.

and easier technique, we investigated the use of needle biopsy and visual inspection of the gross morphology in determining the reproductive stages of *Spisula*.

Spisula of at least 14.3 cm (14.3 to 18.0) in shell length (adults) were collected during the summers of 1996 and 1997 from Menemsha Bay, Martha's Vineyard, Massachusetts, where dual cycles of maturation were suspected. A continuous temperature recorder was also deployed. Animals from other sites were collected during normal MRC activities. Histologic sections of gonads were staged using Ropes's method (4). Staging accuracy of the other two methods was compared to histologic staging. For needle biopsies, an 18-g, 4-cm needle was inserted between the valves about 4 cm from the hinge towards the siphon. Gametes were drawn into a syringe containing 0.5 ml of seawater and mixed. Samples (20 μ l each) were applied to three microscope slides. One slide, designated as a wet preparation, was immediately cover-slipped and viewed under a compound light microscope. On the other two slides, a drop was smeared, air-dried, stained with Giemsa, cover-slipped, and examined. The stages of gonadal development were evaluated on the basis of the quantity and size of the eggs and the quantity (and motility on wet preparations) of the sperm. Three stages of development were distinguished in the biopsy slides; their relationship to

stages identified from histological preparations is given in parentheses: ripe (ripe and advanced late active animals), partially ripe (early active to late active), or spent (spent and partially spent). Gross morphology was examined by cutting through the adductor muscles, removing one shell, and exposing the external surface of the soft body. The gonad was opened with a dorsal to ventral incision to expose the gonads and gonadal fluid.

Examination of both wet and Giemsa-stained biopsies showed that eggs were larger and more abundant in ripe females (Fig. 1A, B). A bilobate amphinucleolus composed of equal-sized lobes (5) was also apparent in mature eggs. Sperm from ripe males was more abundant and more motile than sperm from partially ripe males. Male and female gonads classified as spent showed few eggs and sperm and few aggregations of hemocytes.

Gross morphology showed a distinction between clams in different stages of development. The ripe females had distended body walls. When cut, the gonads were pale pink to salmon pink and extruded large quantities of similarly colored gonadal fluid (Fig. 1C). Ripe males were also distended, with creamy white/tan gonads that extruded white fluid when cut. Clams in early active to late active stages had less swollen bodies, white gonads, and only small to moderate amounts of whitish gonadal fluid. Spent animals had wrinkled, collapsed body walls, little or no gonadal tissue, and no gonadal fluid (Fig. 1D, E).

Examination of clams collected from Menemsha Bay on three dates in 1997 (Fig. 2) indicated that two cycles of gonadal maturation have occurred at that location, possibly as a result of a sustained period of high temperatures and abundant food. Further examination of these two factors in relation to gonadal maturation may help to predict when dual cycles will occur.

Needle biopsy of gonadal tissue provided a nondestructive means of sampling the gonadal tissues, was easily accomplished, and could be examined either wet or stained. Because of its convenience, the technique could be used in the field to ensure that only usable specimens were collected. This investigation thus provides a model for other efforts to conserve declining populations of animals used in research.

We thank Edward Enos and the MRC divers for their support and help.

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Effect of *In Vitro* Culture of Mammalian Embryos on the Architecture of the Zona Pellucida

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The mammalian zona pellucida (zona) is composed of glycoprotein filaments organized in three layers that differ in their orientation and birefringence (1). Relative to the embryo, filaments of the inner zona are oriented radially, whereas filaments of the outer zona are oriented tangentially. The inner and outer layers are separated by a layer of minimal birefringence, suggesting random orientation of filaments in the mid-zona. Before implantation, the blastocyst must hatch from the zona. *In vitro* culture of embryos reduces their rate of implantation, which limits the effectiveness of *in vitro* fertilization for treatment of infertility in humans. We hypothesized that *in vitro* culture may alter zona architecture.

To test the hypothesis that *in vitro* culture of mammalian preimplantation embryos alters the architecture of their zona, female B6C3F1 mice were superovulated, mated, and sacrificed at various times to obtain 6- to 8-cell, compacted morula (c.m.), and blastocyst-stage (b.c.) embryos developed under *in vivo* and *in vitro* conditions. Retardance and azimuth images were generated with a Zeiss Axiovert 100 TV inverted microscope equipped with a 40× Plan Neofluar oil objective, Princeton Instruments (Trenton, NJ) Micromax digital camera, and the CRI (Cambridge, MA) Pol-Scope system (2). Diameters of zona layers were measured with Metamorph (Universal Imaging Corporation, West Chester, PA) at four locations for each embryo. Mean ($\pm$ 1 standard deviation) diameters of the zona layers were calculated and compared to identify effects of *in vitro* development. Significance was tested by *t*-test, Mann-Whitney, or one-way ANOVA (Table I).

In vitro cultured embryos did exhibit thicker zones than *in vivo* cultured embryos at the 6- to 8-cell ($P = 0.004$) and c.m.

stages ($P = 0.001$). The increase in the total thickness of the zona was accounted for by an increase in the inner layer; this thickening first appeared at 6- to 8-cell stage and reached significance at the c.m. stage ($P = 0.001$). The multilayered architecture of the zona persisted until the blastocyst stage, when the zones of *in vitro* and *in vivo* embryos changed to a single layer, with its filaments oriented tangentially. The total thickness of the zona actually increased from the 6- to 8-cell stage to the c.m. stage of *in vitro* embryos, an effect not seen in the *in vivo* cultured embryos. The zona thickness at blastocyst stage was not significantly different between the *in vivo* and *in vitro* groups. Azimuth of zona layers was not affected by *in vitro* culture.

Both mechanical (e.g., embryo expansion and contraction) and chemical (e.g., zona lysins) factors are thought to shape zona architecture during preimplantation development of the embryo. By perturbing the compaction process itself, *in vitro* culture could increase the diameter of the whole zona and the inner zona. Enhanced embryo compaction or delayed expansion would reduce strain on the filaments of the zona and appear as zona thickening at the c.m. stage. If *in vitro* culture enhances compaction of the embryo or delays its expansion, we should observe a reduction in embryo size, measured by Metamorph, relative to *in vivo* cultured embryos at this stage of development. Furthermore, excessively compacted, *in vitro* cultured embryos should exert less strain, measured as retardance by the Pol-Scope, on their zones. *In vitro* culture also could impair the secretion of zona lysins by the embryos, an effect that we would expect to preferentially affect the inner zona layer.

Table I

Effect of *in vitro* development on the thickness of layers of the zona pellucida

Stage of development	Entire zona		Inner layer		Outer + Middle layer	
	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>
6–8 cell	7.50 $\pm$ 0.73 ^{1,4} <i>n</i> = 16	6.78 $\pm$ 0.40 ¹ <i>n</i> = 13	4.05 $\pm$ 0.66 <i>n</i> = 16	3.55 $\pm$ 0.384 <i>n</i> = 13	3.04 $\pm$ 0.45 <i>n</i> = 16	2.85 $\pm$ 0.51 <i>n</i> = 14
Compacted morula	8.54 $\pm$ 0.60 ^{2,4} <i>n</i> = 12	6.65 $\pm$ 0.74 ² <i>n</i> = 10	4.81 $\pm$ 0.79 ³ <i>n</i> = 12	3.32 $\pm$ 0.86 ³ <i>n</i> = 9	3.19 $\pm$ 0.64 <i>n</i> = 12	3.29 $\pm$ 0.72 <i>n</i> = 9
Blastocyst	7.00 $\pm$ 1.379 <i>n</i> = 19	6.72 $\pm$ 0.903 <i>n</i> = 11				

Note: The data presented are the mean thickness (μ m) $\pm$ 1 standard deviation for the number (*n*) of samples indicated.

¹ Significant ($p < 0.004$) by *t*-test.

² Significant ($p < 0.001$) by *t*-test.

³ Significant ($p < 0.001$) by *t*-test.

⁴ Significant ($p < 0.05$) by one-way ANOVA.

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Identification of Phospholipase A₂ and Phospholipase C Activities in Calcium Regulatory Endomembranes of Sand Dollar Cells

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The calcium signal that precedes nuclear envelope breakdown (NEB) (1, 2)—called the pre-NEB Ca²⁺ signal—is composed of nonrandom space-time patterns of Ca²⁺ quantum emission domains (QEDs), acting within microdomains (2-4). Mathematical analysis and modeling of these pre-NEB Ca²⁺ signals has shown that their constituent QEDs are generated locally and act locally; i.e., the agonist used to generate these signals, as well as Ca²⁺ itself, does not diffuse over distances greater than 1 μ m (2-5). Therefore, to generate QEDs, there must be a cellular mechanism whereby calcium can be released in a quantal fashion by an agonist that, once having evoked a release of calcium to the cytoplasm, is quickly rendered incapable of stimulating further calcium release. The action of such an agonist would thus be limited to discrete regions of space and time (2).

This laboratory has previously discovered and demonstrated that leukotriene B₄ (LtB₄) can serve as an agonist of Ca²⁺ release from endomembrane stores *in vivo* and *in vitro* (6, 7) in sand dollar (*Echinarcinus parma*) eggs and mitotic cells. Only the reduced form of LtB₄, not its oxidized form or other products of the arachidonic acid (AA) pathway, will evoke Ca²⁺ release from intracellular stores (6, 7). LtB₄ is notable in that it undergoes spontaneous oxidation under physiological conditions; and oxidized LtB₄ does not evoke Ca²⁺ release from cell stores *in vivo* or *in vitro* (8-10). Therefore, following its enzymatic formation from leukotriene A₄, LtB₄ can act as an agonist and be degraded upon release from its site of action, and any unused LtB₄ would be oxidized before it could diffuse from the region in which it was produced. It has been shown that 1-4-5 inositol triphosphate (IP₃) can also serve as an agonist to evoke Ca²⁺ QEDs (6, 7, 11-13). To better understand the process of Ca²⁺ regulation, we need to know how Ca²⁺ QEDs are generated. One step is to demonstrate that the enzymes that produce these agonists from the phospholipid substrate (i.e., phospholipase A₂ (PLA₂) for AA and LtB₄, and phospholipase C (PLC) for IP₃) are present within the cell and in association with endomembranes that control intracellular calcium signals.

We have now identified PLA₂ and PLC enzyme activities on endomembranes from eggs of *Echinarcinus parma*. Eggs were obtained from mature female sand dollars as previously described (1), and then disrupted by homogenization in a buffer composed of 250 mM sucrose, 100 mM KCl, 10 mM NaCl, 30 mM imidazole, 5 mM MgCl₂, 0.307 mM NaN₃, pH 7.2, on ice. Crude homogenates were overlaid onto sucrose density step gradients and centrifuged at 140,000 $\times$ g, at 2°C, for 3.5 h in a Beckman SW-41Ti rotor (11, 14). We developed spectrophotometric assays for PLA₂ and PLC activities that are adaptations of previously published methods for PLA₂ (15) and PLC (16). Additional assays were performed for glucose-6-phosphate dehydrogenase, a marker enzyme found in the endoplasmic reticulum, and creatine kinase (14, 17). Standard curves for enzyme product were generated for each experiment at *log*/2 and *linear*/2 concentration steps; R² typically >0.99. Triplicate assays were performed for each membrane subfraction. Specific and total activities for the enzyme activities of each subfraction were determined, and the values compared.

The total amounts of endomembrane-associated PLA₂ and PLC activities in eggs are nearly equal. PLA₂ and PLC enzyme activities are differentially distributed among the different endomembrane subfractions isolated from eggs. PLA₂ is present primarily in the less dense subfractions; for instance 90% of the total specific activity for PLA₂ was found in subfractions A1 (40.3%) and A2 (33.2%), 14% in subfraction B, 4% in subfraction C, and 7% in subfraction D. In contrast, the greatest amount of PLC activity (67.8% of total endomembrane-associated specific activity) was found in subfraction D, with the remaining activity distributed among A1 (1.3%), A2 (2.1%), B (28.7%), C (29.4%), and D (38.3%). Thus, PLA₂ enzyme activity is found predominantly in the less dense endomembrane subfractions (A1, A2; 73.5%), whereas PLC enzyme activity is present predominantly in the most dense endomembrane subfraction (D; 67.8%). These data are seen in Figure 1.

PLA₂ acts on phospholipids to yield AA and monoacylglycerols (8), and AA is converted to LtB₄ through the 5'-lipoxygenase branch of the AA pathway. We demonstrated previously that LtB₄ evoked release of Ca²⁺ from endomembranes *in vivo* and *in vitro* (6, 7); this is consistent with a role for LtB₄ in secretory

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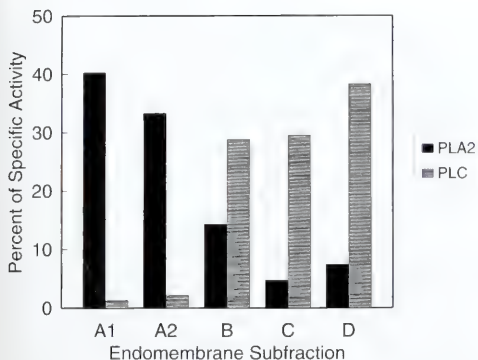


Figure 1. Differential distribution of the percent of total specific activity for phospholipase A_2 and phospholipase C in endomembrane subfractions resolved on sucrose density gradients. Endomembrane subfractions were collected at the interfaces of solutions of different sucrose molarities, i.e., 0.25/1.0 (A interface), 1.0/1.3 (B interface), 1.3/1.5 (C interface), and 1.5/2.0 (D interface); endomembranes at the 0.25/1.0 M sucrose interface were resolved into two bands. The endomembrane subfractions from these different sucrose interfaces were designated A1, A2, B, C, and D, respectively. Spectrophotometric assays for PLA₂ and PLC activities were adapted from previously published methods for PLA₂ (15) and PLC (16).

cells (9). LtB₄ appears to be at least 15-fold more effective than IP₃ in evoking Ca²⁺ release from endomembranes in aequorin-labeled sand dollar eggs (2, 18). It is not clear, at this time, if LtB₄ evokes calcium release by acting (a) directly upon an IP₃-sensitive calcium release channel; (b) through a multistep mechanism upon an IP₃-sensitive calcium release channel; (c) upon some other, possibly undiscovered, type of calcium release channel; or (d) some combination of (a), (b) and (c). We have argued that LtB₄ fits the requirements of an agonist needed for generation of QEDs (6, 7). The endomembrane subfractions that exhibit ATP-dependent Ca²⁺ uptake activity and LtB₄ sensitive Ca²⁺ release, namely A1, A2, and B (11, 14, 17), have the major portion of PLA₂ activity. In contrast, PLC, which acts on phosphatidylinositol to yield IP₃ and diacylglycerol, is present in much smaller amounts on the Ca²⁺-regulatory endomembranes, and resides primarily in the densest endomembrane subfraction.

To evoke Ca²⁺, QEDs an agonist must be generated proximal to sites of Ca²⁺ release and Ca²⁺-dependent action (2). We established earlier that such a signal originates from intracellular Ca²⁺ stores (e.g., endoplasmic reticulum) that are present throughout the mitotic apparatus (1, 2, 11, 14). Rapid (e.g., nonenzymatic) degradation of an agonist to an inactive form

would help ensure the quantal nature of a signaling mechanism using that agent. In addition to LtB₄, IP₃ is known to be an agonist for Ca²⁺ signaling systems (12, 13). In light of the aequorin-based information we have developed, including that described above, IP₃ does not appear to control pre-NEB and other mitotic Ca²⁺ signals (6, 7). Thus, we find that the PLA₂ enzyme activity needed to produce AA (the primary substrate for production of LtB₄) is present within the same endomembrane subfractions that exhibit ATP-dependent Ca²⁺-uptake and LtB₄-sensitive Ca²⁺ release. That these activities can produce equivalent amounts of product, yet are differentially distributed among the endomembranes, may indicate that those enzymes have different roles in controlling Ca²⁺ signaling. These results may also suggest that PLA₂ and PLC produce agonists that act on intracellular Ca²⁺ stores through different mechanisms and pathways. We expect that this hypothesis can be readily extended to other agonist-stimulated, Ca²⁺-dependent cellular processes.

Grant support by NSF (MCB-9602056) is gratefully acknowledged. Aequorin preparations (18) were generously provided by Dr. Shimomura (Marine Biological Laboratory) and his colleagues Drs. Inoué, Musicki, and Kishi. The authors are grateful to the reviewers for their many helpful and well-considered suggestions made in the final preparation of this manuscript.

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Statistical Image Analysis of Spatial and Temporal Patterns in the Pre-Nuclear Envelope Breakdown Calcium Signal

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Mitotic events are signaled by transient elevations in intracellular free calcium ion (Ca^{2+}_i). Nuclear envelope breakdown (NEB) is such a mitotic event (e.g., 1–4); it is preceded by a large signal of Ca^{2+} , composed of several thousand individual events of elevation of Ca^{2+} concentration, i.e., the pre-NEB calcium signal (1, 4). The events in which Ca^{2+} is released to the cytoplasm are seen as bright observable blobs (BOBs) in aequorin-labeled cells; when these BOBs are found to be linked in a nonrandom fashion in space-time they are called quantum emission domains (QEDs) (1, 2, 5–10) that act within microdomains (5, 7, 8). Earlier analyses indicated that pre-NEB Ca^{2+} signals arose from peri-nuclear microdomains, and were neither waves nor exhibited wavelike behavior (8, 11–16). Several independent statistical tests revealed no evidence of a diffusion-related component acting at distances $>1\ \mu\text{m}$ in these pre-NEB signals. Statistical optical models and probabilistic filters demonstrate that the pre-NEB Ca^{2+} signals arise as structured events that differ from global or chain reaction (e.g., Ca^{2+} -induced- Ca^{2+} release) models for Ca^{2+} signaling. The time and duration of these Ca^{2+} signals coincide with the period during which cells require Ca^{2+} signals to accomplish NEB and subsequent mitotic steps. Because waves are reported in various studies (e.g., fertilization [15–21], *Xenopus* mitochondria [22, 23], cardiac myocytes [24]), we have extended our analyses to unfiltered image data sets.

Photons are scattered by cellular components. If that scattering is unbiased (as in eggs and blastomeres) then the measured spatial center of mass (COM) of the scattered BOBs over a sufficient time period should be an unbiased estimate of the location of the actual COM of the corresponding photon emissions. The COM is the weighted center of for all BOBs detected in a cell during a given period of time. We have measured the COM for pre-NEB Ca^{2+} signals of mitotic sand dollar cells. This approach is advantageous in that we compute global statistics of the whole data set, and no filtering or preselection of data is necessary. Temporal distribution of BOBs shows a fast rise to peak amplitude and a slower return to baseline. We determined the COM of signal activity for Ca^{2+} signals of pre-NEB and for wounds at the cell surface. The trajectory of pre-NEB COM begins distal to the nucleus, then moves to a juxta-nuclear position; waves due to wounds exhibit linear tracks. Any transverse wave motion of the Ca^{2+} signal would have appeared as an oscillation in the COM trajectory. The highest frequency detectable was 15 Hz, i.e., analysis of single 33 ms frames per event; no waves were detected at sampling frequencies between 0.05 Hz and 15 Hz.

Regular, identifiable patterns of Ca^{2+} signals should yield to Fourier analysis, i.e., in the temporal domain. Indeed, the Fourier spectrum of BOBs indicates nonrandom frequencies within pre-NEB Ca^{2+} signals (25). An analytical approach we have used that sidesteps the scattering issue is to perform a temporal Fourier transform on all the detected luminescent events. In this way, if there are any global-intensity oscillations in the event emissions, they should be detected by high peaks in the amplitude of the Fourier spectrum. A typical Fourier spectrum for events in a pre-NEB Ca^{2+} signal shows a frequency peak at 6 Hz; similar peaks at 0.2 Hz and 0.4 Hz are also observed. Our method is a form of the Hough transform in which each event is tallied in accumulator-space (i.e., the set of temporal pixel locations) according to its time and a distance-weighting function in space. This is a variation of our probabilistic filtering algorithm; the distance-weighting function is a Gaussian with a similar form to the estimated scattering statistical point spread function. A maximum in accumulator-space will provide evidence for that pixel location being the origin of many of the events that originated in that space. Once these centers are identified by peak detection in accumulator-space, statistical scale-space clustering methods are used to identify statistically significant locations. To date, we have obtained a number of centers at different temporal scales (from 1 to 8 frames per sample space); scale-space grouping of these events is in progress. This analysis represents the first step in locating local Ca^{2+} sources, i.e., within microdomains. Once statistically significant centers are identified, we track their motion and frequency response using our event-tracking algorithms. From these images we have found that there are many coincident matches between the two images; furthermore, all peaks of QED activity reside within the cell.

Our early efforts to process luminescence data consisted of identifying spatial repetitions with temporal constraints. Analyses of some of the pre-NEB Ca^{2+} signal records proved inconclusive due to the high density of luminescent events coupled with the inherent uncertainty in an event's spatial location; i.e., the very brightest signals often showed overlapping QEDs, which were difficult to resolve and then analyze as discrete events. In contrast, our current methods identify a small number of the most likely centers of Ca^{2+} release by considering all detected events in a small time interval. Correlations between different time intervals provide further statistical evidence for emission centers. Now that fewer locations with higher spatial certainty have been identified, our original grouping algorithms can be applied to further characterize the activity. The deterministic nature of these signals indicates that the paradox of Ca^{2+} signaling (i.e., calcium being both "universal" and specific and selective) is resolved through structure inherent in these signals (4). We hypothesize that this paradox of Ca^{2+} signaling is re-

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solved by the appearance of structured, nonrandom patterns of quantum emission domains (QEDs) arising within microdomains.

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Apoptosis and Tissue Regression in the Marine Sponge *Microciona prolifera*

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Cell recognition and differentiation in the marine sponge *Microciona prolifera* is mediated by a highly glycosylated and sulfated adhesive proteoglycan (MAF). During growth and differentiation, sponge presents a variety of cell types derived from stem cells (archeocytes) and organized in the context of a fibrosiliceous framework. Adult differentiated sponges generate free-swimming larvae, usually in late June or July, that ultimately become sessile and mature into adult forms. In the fall and winter, there is a decline in cells and cell types, a loss of coloration, and a collapse of sponge chambers. The cells that remain, primarily archeocytes, become encased in relatively

dense supporting tissue that offers protection from adverse conditions (1). These changes are the likely result of programmed cell death (2, 3, 4), to which the term apoptosis was given by Kerr *et al.* (5). The results of the present study support this possibility.

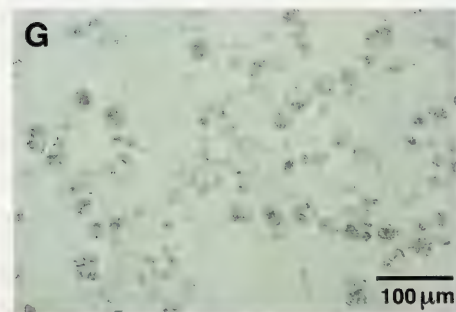
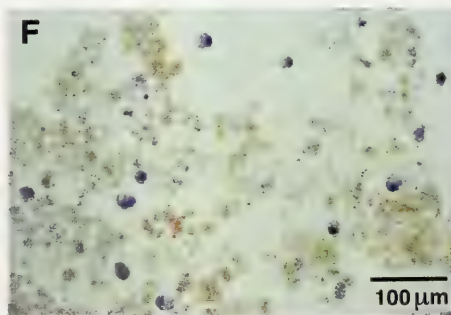
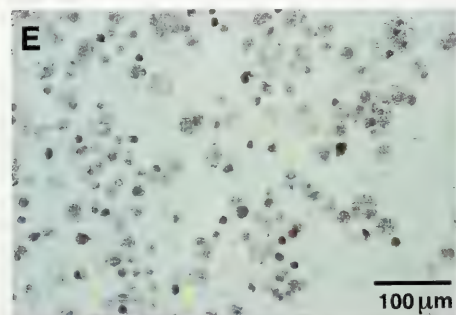
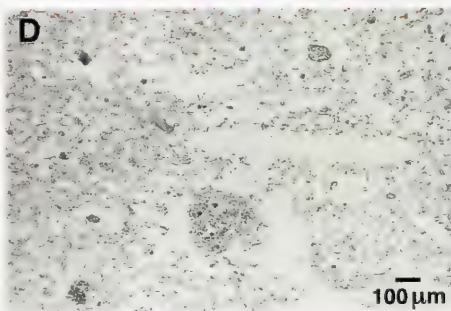
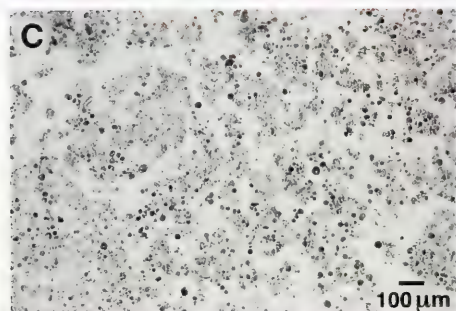
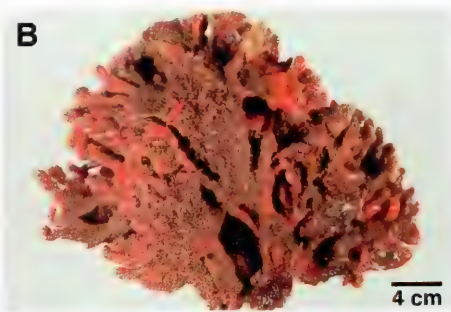
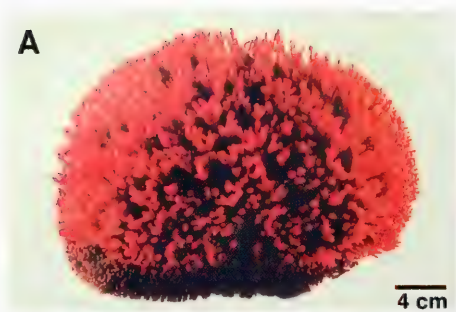
To determine apoptosis at the cellular level, we carried out a TUNEL assay (6) for analysis of labeled DNA strand breaks that could be analyzed by light microscopy. The name of the assay stands for a terminal deoxynucleotidyl transferase-mediated dUTP nick end label that can bind to 3' OH ends of disrupted DNA. Paraffin sections (5 μ m) were deparaffinized and treated successively with proteinase K for 30 min, endogenous peroxidase blocking solution for 30 min and then permeabilized with 0.1% Triton X100. Nonspecific reactive sites were blocked. The TUNEL mixture was reacted with cells for 1 h and the sections were then treated with sheep anti-fluorescein

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Figure 1. (A) Gross appearance of summer (July) sponge. (B) Winter (February) dormant sponge—note pigment loss and collapse of chambers. (C) Stained section of branch from summer sponge indicating high degree of cellularity and many stained cells. (D) Winter sponge shows a small number of islands with few stained cells surrounded by a primarily fibrous matrix. (E) TUNEL reaction on summer sponge showing nuclear DNA as brownish red; 1, 7 = phagocytosed apoptotic cells; 2, 5 = small apoptotic cells; 3, 4, 6 = closely adherent macrophage-type apparently healthy cells and small apoptotic cells. (F) TUNEL reaction of winter sponge. A small population of healthy stem cells with counterstained blue nuclei is surrounded by irregular masses of brown apoptotic debris. (G) Counterstained control section in which TUNEL reagent was omitted.



horseradish conjugate and 3,3' diaminobenzidine (DAB) or 3-amino 9-ethyl carbazole (AEC) substrate to develop the color. Counterstaining was carried out using hematoxylin.

The gross appearance of *Microciona* adult summer sponge and of dormant or winter sponge is shown in Figure 1A and 1B, respectively. The brown color of dormant sponge indicates that pigment cells are virtually absent. The tissue is more compact than that of summer sponge. Stained microscopic sections derived from summer (1C) and winter (1D) sponge tissue were markedly different in cellularity, as demonstrated by Alcian blue-iron diamine at pH 2.5. Apoptotic cell patterns, as revealed by the TUNEL assay, were observed in sections derived from summer and winter sponge. DNA strand breaks indicated by the TUNEL reaction occurred in both summer and winter sponge sections. Winter sections displayed large fragments of apoptotic debris intermingled with an almost uniform population of large counterstained (i.e., non-apoptotic) cells, which we interpret as archeocytes or stem cells. Summer sponge contained a mixture of differentiated cell types and archeocytes. The nuclei of some large cells stained normally, although the cytoplasm contained phagocytosed apoptotic cells or cell fragments. The nuclei of other noningested cells, presumably nonviable, were apoptotic. Both kinds of cells, together, constituted >30% of the total cell population (see Figs. E, F, and G).

Winter sponge gave the expected picture of a small, apparently healthy stem cell population surrounded by masses of apoptotic material. Since *Microciona* sponge is a non-germinating sponge, we surmise that this substance may function as a protectant for cells that will eventually form differentiated progeny. However, we were surprised by the extent of apoptosis in summer sponge. The combination of particle ingestion by

these filter feeders, the generation of MAF and its cell-differentiating effects, and endogenous cell turnover allowing for the disposal of worn out cells may contribute to the extent and heterogeneity of the apoptotic forms noted. This could be similar to mammalian cell disposal; for example, phagocytosis of senescent erythrocytes by Kupffer cells of the liver (7). The interesting microscopic picture observed in dormant tissue may reflect the result of up-regulation of apoptotic networks whose reversal may presage cellular regeneration, increased synthesis of MAF, differentiation, and larval development. The unique and divergent character of winter vs. summer sponge provides opportunities to chemically characterize cell suicide markers (8) on the one hand and cell growth factors on the other, and in this way provide opportunities for studies of their regulation. A more accurate assessment of the timing of these phenomena may be possible with an increased frequency of sampling, a study now under consideration.

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Cell Localization of Lectin Binding in *Microciona prolifera* Sponge

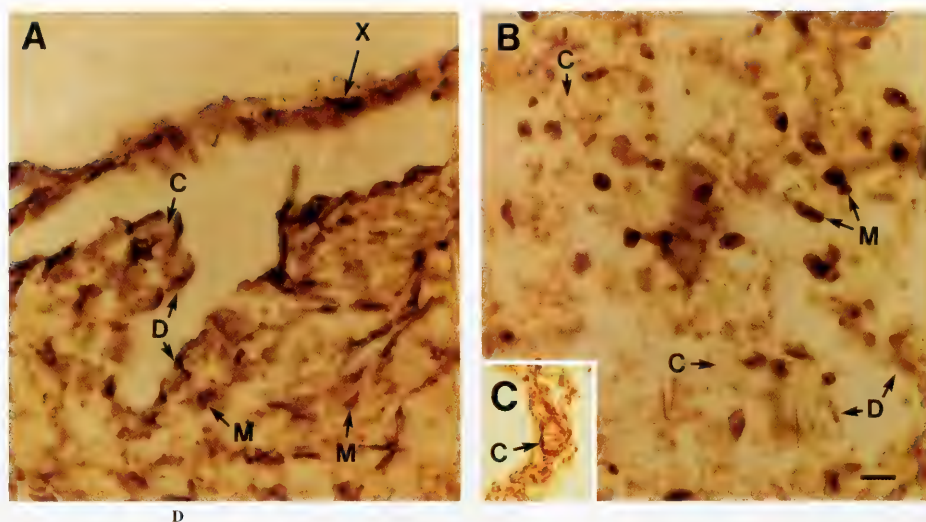
Kristin M. Hudock, Ashi Hafeez, and Jane C. Kaltenbach
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Our previous studies have demonstrated general lectin staining and sulfate masking of sugars in *Microciona prolifera* cell pellets; however, we were unable to assign lectin binding patterns to specific cell types. Lectin specificities on sponge branches may yield important information within the context of sponge structure, in contrast to cell pellet-derived cells where lectin histochemical staining is an important guide toward further biochemical definition (1). The purpose of the present study was to identify specific sponge cells and their patterns of lectin binding. We have used sections cut from *Microciona* sponge branches to preserve cell relationships, and we have used lectin histochemical probes to localize the sugar ligands.

Sponge branches were fixed in formalin, embedded in paraffin, and sectioned at 8–12 μ m. The sections were incubated with the following horseradish peroxidase (HRP)-conjugated lectins (final concentration 0.025 mg/ml): wheat germ agglutinin (WGA) with affinity for N-acetyl-glucosamine (GlcNAc),

soybean agglutinin (SBA) for N-acetyl-galactosamine (GalNAc), concanavalin A (Con A) which binds to α -mannopyranoside (Man), *Ulex europaeus* (UEA-1) for α -L-fucose, and peanut agglutinin (PNA) with affinity for β -galactose (Gal). As a control, each lectin was reacted with its inhibitory sugar (0.2 M) before being applied to the tissue.

Sponges lack organ systems, but they do possess epithelial tissue and distinct types of cells (2). In the sections of sponge branches we were able to identify the following epithelial cells: exopinacocytes (covering the outer sponge surface), endopinacocytes (lining the water canals), and choanocytes (lining choanocyte chambers in the canal system). However, we were unable to differentiate between the cell types in the mesohyl (between the pinacocyte layers) including rhabdiferous cells, archeocytes, and gray cells. Each cell type had a characteristic lectin binding pattern (Fig. 1). For example, endopinacocytes stained intensely with WGA, moderately with Con A, but did not stain with SBA,



Lectin	Exopinacocytes	Endopinacocytes	Choanocytes	Mesohyl Cells
WGA	+++	+++	++	+++
SBA	+	—	+	+++
UEA-1	++	—	—	+++
Con A	+++	++	+++	+
PNA	+	—	++	+++

Figure 1. Comparison of lectin binding in specific cell types in sections of *Microciona prolifera* sponge branches. A: Incubated with WGA (specific affinity for GlcNAc). Staining is intense in exopinacocytes (X) covering the surface of the sponge, endopinacocytes (D) lining the water canals, and oval shaped mesohyl cells (M), but moderate in the flagellated choanocytes (C) of the choanocyte chambers. B: Incubated with UEA-1 (specific affinity for Fuc). Staining is intense in the mesohyl cells (M), but absent from endopinacocytes (D) and choanocytes (C). C: Incubated with Con A (specific affinity for Man). Staining is intense in the choanocytes. D: Semiquantitative evaluation of lectin staining. (+++) intense; (++) moderate; (+) weak; (—) absent. Scale bar = 100 μ m.

PNA, or UEA-1. Choanocytes displayed a particular affinity for Con A and less so for other lectins, but mesohyl cell stained intensely with most of the lectins. Future experiments on sponge branches should indicate whether desulfation may enhance weak or absent cell specific lectin staining, as previously observed in cell pellets (1).

The cell surface sugar residues, as defined by these lectin probes, may bind to the aggregation factor (MAF) or to endogenous lectins; they may also function as adhesins towards bacteria, algae, etc. (3, 4, 5, 6). The use of plant lectins as probes to define sugar patterns in sponge cells may be helpful in further studies of intrasponge cellular relationships as well as symbiotic relationships between sponge and heterologous cells.

The authors wish to thank Dr. Tracy L. Simpson for his help with cell identification and Dr. William J. Kuhns for his guidance, both with research and preparation of the manuscript.

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Binding of Hyaluronic Acid to Cellular Receptors of the Marine Sponge *Microciona prolifera*William J. Kuhns¹, Michael Ho¹, Max M. Burger², and Eva Turley¹

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The intercellular matrix (MAF) of the marine sponge, *Microciona prolifera*, is a large proteoglycan of ca. 20×10^6 molecular weight comprised of multiple subunits. Each of these is highly glycosylated and sulfated and contains about equal amounts of protein and carbohydrate. There is a protein core from which radiate many arms with a high carbohydrate content. Subunits are linked to each other via carbohydrate-carbohydrate bonds, two epitopes of which of which have been chemically defined (1). Hyaluronic acid (HA) is a component of MAF, but until now its functional role has been unclear. Recently an MAF peptide has been cloned by Fernandes-Busquets *et al.* A portion of the peptide contained an amino acid sequence that was thought to be a binding motif for HA (2). This finding raised the possibility that HA might facilitate adhesion between MAF and cell surface receptors in *Microciona*.

In this study, we used biotinylated HA (3) as a probe directed towards the membranes of *Microciona* sponge cells that had been chemically dissociated in accordance with earlier protocols (4, 5), pelleted from suspension by centrifugation, fixed in formalin, embedded, and sectioned at 5 μ m. Non-specific reactive sites were blocked with buffered 1% bovine serum albumin. Sections were stained with biotinylated HA for 1 h at room temperature and washed; color development was optimized with streptavidin-horseradish peroxidase conjugate followed by 3,3'-diamino benzidine (DAB) for 4 min. Control staining was carried out in parallel using biotinylated HA that had been pre-treated with hyaluronidase. The possible masking effect of sulfate was determined by chemical desulfation of prestained sections as described previously (6).

Figure 1B demonstrates a strong peroxidase staining pattern over, and at the periphery, of most cells on preparations that had been chemically desulfated. This effect is especially pronounced on the larger cell forms, probably archeocytes. Peroxidase staining was less intense in cell preparations that had not been desulfated (1A), indicating that sulfate is masking HA binding sites. No peroxidase staining was observed in the hyaluronidase treated sections (1C). To identify surface HA receptors, we carried out immunohistochemical staining using anti-

bodies against several HA binding proteins. One of them, a monoclonal antibody towards a receptor for HA associated with motility (RHAMM) (7), demonstrated peroxidase staining in a subpopulation of *Microciona* cells (1D).

This is the first demonstration of immunochemical staining for HA receptors on *Microciona* sponge cells, and also the first indication that RHAMM occurs in a lower invertebrate. We suggest that the presence of HA in MAF, coupled with the presence of a potential HA binding sequence in its core protein may allow for MAF-cell receptor binding via MAF-HA linkages. Thus, linearized polymers of the disaccharide glucuronic acid and N-acetylglucosamine (HA) might possess a bridging function, or be interspersed with other sugars, but with HA proximal and distal to the protein core. Sulfated ligands may possess a regulatory role in HA—cell receptor binding (8, 9). Direct HA binding studies using synthetic constructs derived from MAF core protein and from RHAMM or other candidate receptor molecules will be important in the assessment of sulfated products as possible binding inhibitors.

The authors thank Dr. Jane Kaltenbach and Kristin Hudock for help in the preparation of the photographs, and Michelle MaCaffery for expert assistance in preparing microscopic sections.

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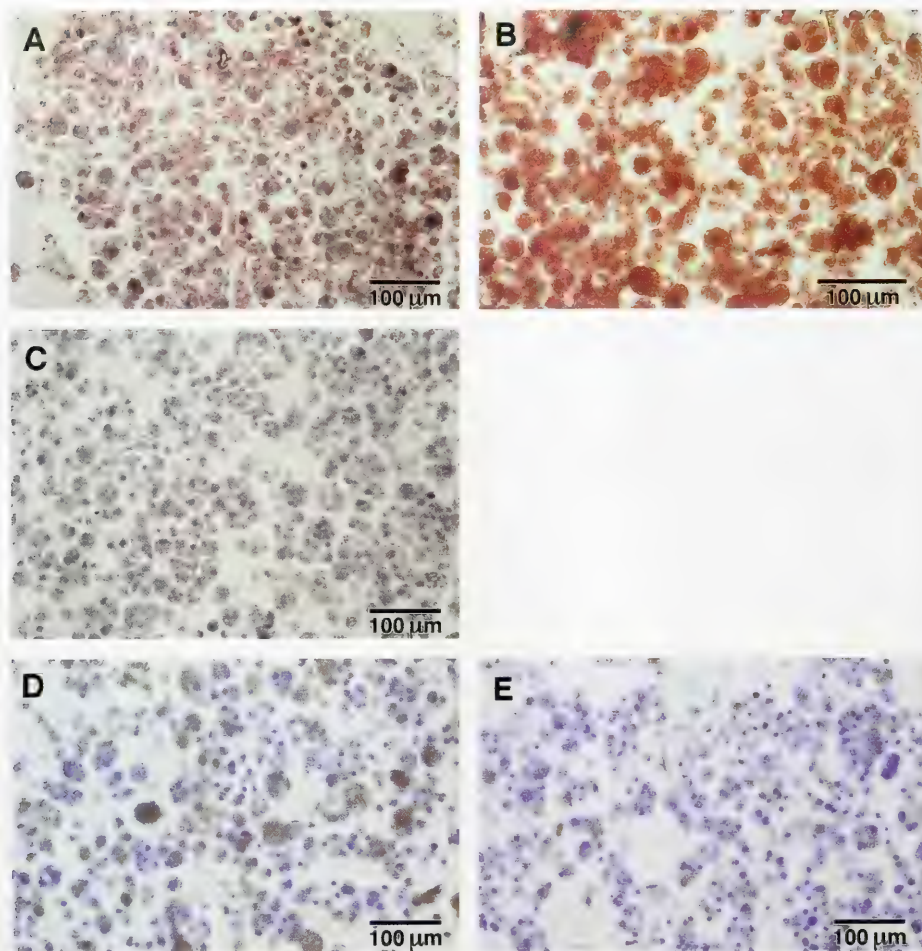


Figure 1. (A) Section (5 μ m) of *Microciconia* cell pellet stained with biotin labelled hyaluronic acid (BHA)-streptavidin horseradish peroxidase conjugate. Staining is noted in a high proportion of cells, and in many of them is stronger at the periphery. (B) Section of a pellet which had been chemically desulfated before staining. The intensity of peroxidase staining is greater than that of non-treated section. (C) Control section in which hyaluronidase was admixed with BHA and incubated for 1 h at room temperature before staining. No peroxidase staining is observed. (D) Section of pellet stained with peroxidase using monoclonal antibodies raised to RHAMM; note the stain in a subpopulation of *Microciconia* cells. (E) Control preparation in which antibody was omitted.

Experimental Analysis of Tentacle Formation in the Ctenophore *Mnemiopsis leidyi*

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The use of intracellular lineage tracers in conjunction with simple operative techniques has been instrumental in establishing the fates of identified blastomeres during development and regeneration. By injecting the fluorescent lineage tracer DiI into identified blastomeres of the lobate ctenophore *Mnemiopsis leidyi*, we recently showed that the eight rows of locomotory comb plates characteristic of the Ctenophora are derived, not from one (1, 2), but from two distinct embryonic cell lineages, the m_1 as well as the e_1 micromeres (3). Killing e_1 micromeres at the 16-cell stage leads to the complete absence of comb plate formation during the embryonic period (3–5); therefore, the e_1 micromere lineage is required to induce comb plates from m_1 lineage descendants. This “organizing activity” of distinct cell lineages (e_1 descendants) is surprising given the widely touted “mosaic” nature of ctenophore development (2, 6, 7).

The e_2 blastomere lineages may also serve as “signaling centers” in ctenophore embryogenesis, as suggested by investigations of tentacle formation. Ctenophore tentacles grow out from tentacle bulbs located on opposite sides of the animal and are used to capture prey (Fig. 1c). The tentacle apparatus is composed of an ectodermal component that invaginates to meet an outpocketing of the endoderm. This tentacle bulb subsequently gives rise to the external tentacle strand that possesses sticky colloblasts. Early marking experiments with chalk particles suggested that the two tentacles of the Mediterranean ctenophore *Bolina hydatina* are derived solely from the e_1 micromeres (2); but tentacles fail to form when either e_1 or e_2 micromeres are deleted (5). Thus, if e_2 cells make no contribution to tentacles, they must provide some cue to the cells that generate the tentacles.

We have reinvestigated the origins of tentacles in *M. leidyi* using the intracellular injection of DiI (3, 9) and have found that tentacles are not derived solely from the e_1 lineage, but are generated from five distinct embryonic cell lineages derived from two adjacent quadrants (Fig. 1a). The ectodermal compo-

nent is derived from the e_1 , m_1 , e_2 , and e_3 micromere descendants while the muscular core of the tentacle is normally derived from mesendodermal cells of the 2M lineage (8). This paper describes cell deletion experiments investigating the role that cell interactions play in tentacle formation.

Figure 1b indicates the results of a series of experiments in which blastomeres known to contribute to the tentacles were deleted with fine glass needles during the 8- to 32-cell stages. Operated embryos were grown for 24–48 h in filtered seawater, at 18–20°C, in gelatin-coated dishes. Each tentacle is made by two adjacent quadrants of the embryo; therefore pairs of identical cells were deleted in most experiments. Tentacles were identified by the presence of both endodermal outpocketings and coordinated ectodermal proliferation. In some cases, it was clear that the tentacle on the operated side had not developed to the same extent as the contralateral control tentacle.

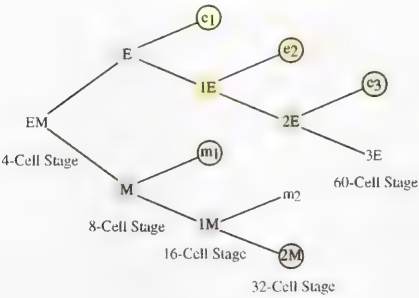
The results set out in Figure 1b clearly show that no single lineage of cells is responsible for the normal formation of tentacles. The destruction of two adjacent, and even all four (data not shown) e_1 cells results in tentacle formation on the operated side. Likewise, tentacles formed in embryos from which adjacent e_2 cells had been ablated. Due to the locations of the cells, generating embryos from which only two M macromeres were deleted at the eight-cell stage was extremely difficult. In a few cases we managed to destroy two adjacent M macromeres, and one single E macromere, and in most cases these formed tentacles. The destruction of the m_1 cells, the 2M cells, or both pairs does not prevent tentacle development. We noted that destroying the 2M blastomeres had no effect on tentacle contractility, indicating that some other lineage of cells generates the muscular core, which is normally derived from the 2M cells. Destruction of both e_1 and e_2 cells eliminated tentacle formation. Ablation of the cells that give rise to e_1 and e_2 , the E blastomeres, also leads to the loss of tentacles. These findings indicate that the e_1 and e_2 cells play an important role in organizing tentacle development.

Tentacle formation appears to be complex, with cells of different embryological origins contributing to the production of

Figure 1. The four-celled embryo contains four “EM” blastomeres that generate the four embryonic quadrants. (1a) Partial cell lineage diagram of an individual EM cell showing the names of each daughter cell through the 60-cell stage. The blastomeres that contribute directly to tentacle formation are enclosed with black circles. The colors identify specific cells shown in the diagrams in 1b. Subsequent divisions of the micromeres (lowercase letters with subscripts) are not shown. (1b) Table recording the results of experiments in which specific blastomeres were killed. The numbers in parenthesis indicate the total number of cells ablated. Diagrams show their locations in the embryos. The total number of operations prepared as well as the number that formed a tentacle on the operated side are also shown. The unoperated contralateral side always developed normally and served as an internal control. (1c–e) Differential interference contrast micrographs of 32–48 h-old cydippids of *M. leidyi* seen in lateral view. The mouth is facing the bottom and the apical organ (ao) is oriented towards the top. (1c) Normal control cydippid with two tentacles (m). (1d) Cydippid in which two adjacent e_2 micromeres were killed at the 32-cell stage. Both tentacles are present. (1e) Cydippid in which two adjacent e_1 micromeres (at the 16-cell stage) and two adjacent e_2 micromeres (at the 32-cell stage) were killed on the same side of the embryo. No tentacle formed on the operated side.

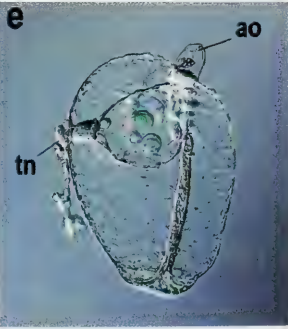
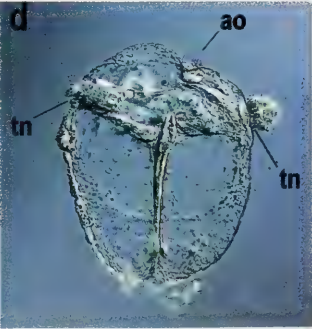
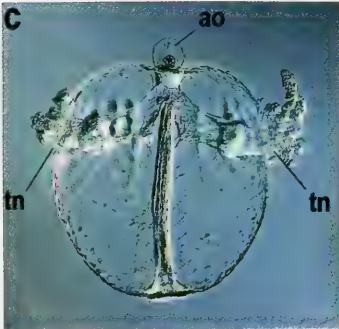
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a



b

Deleted Cells	Diagram	Number Scored	Tentacle Present / Absent	Deleted Cells	Diagram	Number Scored	Tentacle Present / Absent
-E(2)		11	0 / 11	-1M(2)		3	3 / 0
-E(2)&-M(2)		18	0 / 18	-e1(2)&-m1(2)		17	16 / 0
-E(2)&-M(1)		7	0 / 7	-e2(2)		19	19 / 0
-E(1)&-M(2)		11	9 / 2	-2E(2)		36	36 / 0
-e1(2)		15	15 / 0	-2M(2)		34	33 / 1
-1E(2)		33	33 / 0	-e1(2)&-e2(2)		25	0 / 25
-m1(2)		16	16 / 0	-e1(2)&-2E(2)		12	9 / 3



these structures. Our results do not confirm the less extensive experiments performed by Farfaglio (5) on the Mediterranean ctenophore *B. hydatina*. Although earlier workers, using the chalk particle method of following cell lineages, may have missed the contributions of additional cell lineages involved in tentacle formation, we would not expect that the inductive role of the e_2 micromeres on tentacle formation would be so drastically different. Perhaps there are species differences in the roles of identified blastomeres in tentacle formation. One possible explanation may be that the putative inductive role of the e_2 micromeres, which are required for the formation of tentacles in *B. hydatina*, is shared by both e_1 and e_2 micromere descendants in *M. leidyi*.

The authors thank the generous community of the Marine Biological Laboratory and dedicate this work to the memory of Dr. Sebastian Beroë. J.J.H. was supported by a Sebastian Beroë Fellowship. M.Q.M. was supported by NSF and as an Erik B. Fries and MBL Associates Fellow.

Reference: *Biol. Bull.* 193: 247–248. (October, 1997)

Phylogenetic Analysis of the 5-Aminolevulinate Synthase Gene

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All living organisms require tetrapyrroles, usually as heme, chlorophyll, or both. Heme serves as the prosthetic group in a number of important proteins, including hemoglobin, myoglobin, catalase, peroxidase, and cytochrome c, while chlorophyll is used in chloroplast photosynthesis. Formation of tetrapyrroles in all organisms begins with the synthesis of 5-aminolevulinic acid (ALA), the first committed intermediate along the pathway. The enzyme 5-aminolevulinate synthase (ALS) catalyzes the condensation of succinyl-CoA and glycine to yield ALA in a variety of species including animals, fungi, some protozoa and the α -purple bacteria (ALS is reviewed in reference 1). In contrast, ALA in plants and most other bacteria is made from glutamate by an entirely different biosynthetic pathway involving three enzymatic steps and a glutamyl-tRNA intermediate (2).

ALS genes have been sequenced in a variety of vertebrates and bacteria, one protist, and four fungi (see Fig. 1 for GenBank accession numbers). Several interesting features of the ALS gene have been identified (1). The gene is highly conserved in species ranging from bacteria to mammals. About two-thirds of the sequence shows 60–80% amino acid conservation in protein alignments. Two forms of the gene are present in vertebrates: the housekeeping form (H-ALS) is expressed in almost all cells, whereas the erythroid form (E-ALS) is expressed exclusively in differentiating red blood cells. The H and E forms of the gene are regulated by separate mechanisms (1). Heme-regulatory elements have been identified in mouse E-ALS (3), and putative identifications have been made in the H- and E-ALSs of other mammals. Iron-responsive elements (IREs) have been identified in the E forms of several vertebrate genes.

We are sequencing the ALS gene from a variety of represen-

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tative invertebrate and vertebrate species with the goal of generating a representative phylogenetic ALS gene tree. Recently we completed the sequences from beluga whale (*Delphinapterus leucas*) (H form only), cuttlefish (*Sepia officinalis*), and horseshoe crab (*Limulus polyphemus*). In addition, we have a partial sea urchin (*Strongylocentrotus drobachiensis*) sequence and we have begun work on blood and solid tissue from both the annelid *Glycera dibranchiata* and hagfish (*Myxine glutinosa*). In each instance, we first prepare mRNA and then perform two rounds of reverse transcriptase-polymerase chain reactions (RT-PCR) with degenerate primers to obtain two overlapping partial ALS sequences. We then use rapid amplification of cDNA ends (RACE) to obtain the 5'- and 3'-ends of the gene, along with some untranslated nucleotides on either end. Because the information acquired from the RACE products overlaps with that obtained by RT-PCR, all positions amplified by degenerate primers can be unambiguously identified. The complete sequence of each gene is then aligned with all other known ALS sequences, and phylogenetic analyses are performed on conserved regions of the sequences using parsimony, distance, and maximum likelihood methods.

Our sequences for the ALSs of beluga whale, cuttlefish, and horseshoe crab show a very high degree of similarity to other known ALSs. Each new sequence contains several putative heme-regulatory elements, suggesting that, as in some other animals (3), heme is probably an important regulator of ALS activity in these three species. Our current analyses of *Glycera* and hagfish ALS sequences from blood and solid tissue should inform us further about the distribution of the heme-regulatory elements and IREs among various organisms.

Alignment of our sequences with those previously determined

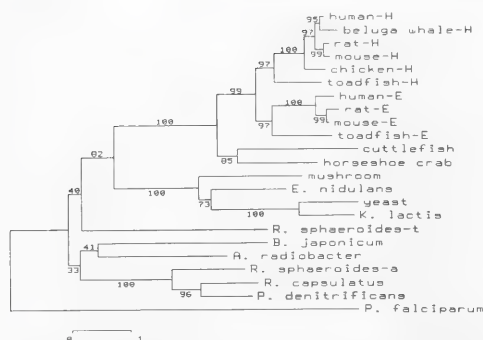


Figure 1. Unrooted protein neighbor-joining tree (using gamma, $\alpha = 2$), generated using MEGA, version 1.02 (reference 5), with bootstrap values (500 replicates) indicated. The scale bar represents 10 changes per 100 amino acid positions. The taxon labels are defined as follows (Genus species, GenBank accession #): human-H (Homo sapiens, X56351), beluga whale-H (Delphinapterus leucas, new), rat-H (Rattus norvegicus, J04044), mouse-H (Mus musculus, M63245), chicken-H (Gallus gallus, X02827 M24366), toadfish-H (Opsanus tau, L35915), human-E (Homo sapiens, X56352), rat-E (Rattus norvegicus, S65966), mouse-E (Mus musculus, M63244), toadfish-E (Opsanus tau, L02632), cuttlefish (Sepia officinalis, new), horseshoe crab (Limulus polyphemus, new), mushroom (Agaricus bisporus, Z50096), E. nidulans (Emmericella nidulans, X64170), yeast (Saccharomyces cerevisiae, M26329), K. lactis (Kluyveromyces fragilis, X92944), R. sphaeroides (Rhodobacter sphaeroides, L07489), B. japonicum (Bradyrhizobium japonicum, M16751), A. radiobacter (Agrobacterium radiobacter, see reference 6), R. sphaeroides-a (Rhodobacter sphaeroides, L07490), R. capsulatus (Rhodobacter capsulatus, X53864 J04825), P. denitrificans (Paracoccus denitrificans, U12508), and P. falciparum (Plasmodium falciparum, L46348).

was straightforward as the gene has a highly conserved C-terminus. In our phylogenetic analyses about 350 amino acid residues or their corresponding nucleotides were included in the calculations. These residues, which account for about half the ALS coding sequence in mammals and up to almost 90% of it

in bacteria, correspond to well-conserved regions of the gene. A representative distance tree based on amino acid sequences is presented in Figure 1. Other methods of phylogenetic analysis produced similar gene trees with one major difference; the branching patterns within the bacterial species and relative to *Plasmodium falciparum* varied between methods. Similar trees resulted whether amino acid or nucleotide sequences were used in the phylogenetic analyses.

One of the most striking features of our tree is the clear monophyletic groupings of the H forms and the E forms of ALS, which suggests the possibility of a gene duplication event. In addition, the tree shows a clear separation of protostomes (represented by cuttlefish and horseshoe crab) from deuterostomes (represented by the chordates). To determine more precisely when the H and E duplication occurred, we are analyzing the ALS genes of sea urchin, an early deuterostome, and hagfish, an early vertebrate with red blood cells. In addition, examining ALSs from blood and solid tissue of *Glycera*, a protostome, should show whether annelids have both H and E forms of the gene. Such information will help us understand both the timing of the H and E duplication and the evolution of red blood cells.

Finally, our tree bears some significance on the endosymbiotic origin of mitochondria. The tree shows that ALSs in unicellular and multicellular eukaryotes are closely related to those in the α -purple bacteria, which have been suggested to be "the closest contemporary eubacterial relatives of mitochondria" (4). Because plants do not use ALS to synthesize ALA, our study raises the question of whether mitochondria in plants and animals originated from the same or different endosymbiotic events.

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Reference: *Biol. Bull.* 193: 248–250. (October, 1997)

Phylogenetic Analysis of Olfactory Receptor Genes from Mudpuppy (*Necturus maculosus*)

Qiao Zhou¹, Gregory Hinkle², Mitchell L. Sogin², and Vincent E. Dionne¹

Since the original cloning of a G-protein coupled, 7-transmembrane domain receptor multigene family from rat olfactory neurons (1), the assemblage has been expanded to a superfamily that now includes homologous sequences from several mam-

mals (rat, mouse, dog, human), one bird (chicken), one amphibian (*Xenopus laevis*), and two teleost fish (catfish, zebrafish). Although a definitive functional assay of the biological function of these receptors is still lacking, the diversity of the putative

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ligand binding sites, the high level of expression in olfactory neurons, and the large size of the gene superfamily suggests that they may be olfactory receptors. In this study, we cloned members of the olfactory receptor genes from a permanently aquatic amphibian, the mudpuppy (*Necturus maculosus*), and conducted a phylogenetic analysis of olfactory receptor genes from major vertebrate groups.

Eighty previously published partial and full length olfactory receptor gene sequences from rat, mouse, human, dog, and catfish were aligned using the PileUp program of the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc.). From two highly conserved regions in transmembrane domains 3 and 7, a pair of degenerate primers were designed:

Tm3 5'-TGGCITAYGAYMGITAYGKGC-3'

Tm7 5'-TADATRAAIGGRTTIARCAT-3'

Total RNA was extracted with an Ultraspec RNA Isolation System (Biotec Inc.) from freshly dissected mudpuppy nasal tissue; it was treated with RQ1 RNase-free DNase (Promega), and converted to first strand cDNA with Superscript II Reverse Transcriptase (Gibco BRL). PCR was performed with an Ericomp thermocycler; the following parameters were used: 94°C for 2 min

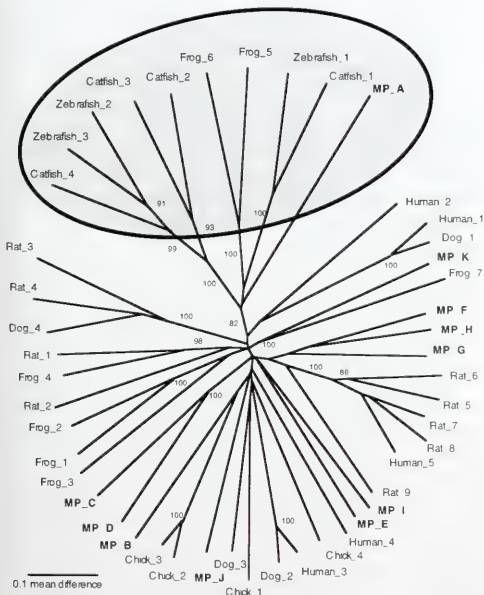


Figure 1. A phylogenetic tree derived from a neighbor-joining analysis of olfactory receptor protein sequences. Bootstrap values greater than 80% are indicated. The accession numbers for the sequences are listed in the table below. The shaded region includes all fish sequences in the dataset as well as two frog and one mudpuppy sequences that consistently cluster together.

Title	Genbank accession	Title	Genbank accession
Dog	<i>Canis familiaris</i>	Rat	<i>Rattus norvegicus</i>
Dog_1	U53681	Rat_1	M64376
Dog_2	U53679	Rat_2	M64377
Dog_3	U53680	Rat_3	M64378
Dog_4	U53682	Rat_4	M64381
Catfish	<i>Ictalurus punctatus</i>	Rat_5	M64386
Catfish_1	L09220	Rat_6	M64387
Catfish_2	L09217	Rat_7	M64388
Catfish_3	L09218	Rat_8	M64391
Catfish_4	L09223	Rat_9	M64392
Chicken	<i>Gallus gallus</i>	Frog	<i>Xenopus laevis</i>
Chick_1	X94742	Frog_1	Y08349
Chick_2	Z79585	Frog_2	Y08350
Chick_3	Z79588	Frog_3	Y08351
Chick_4	Z79592	Frog_4	Y08353
Human	<i>Homo sapiens</i>	Frog_5	Y08345
Human_1	U56421	Frog_6	Y08347
Human_2	X64995	Frog_7	Y08344
Human_3	U56420	Zebrafish	<i>Danio rerio</i>
Human_4	X80391	Zeb_1	U42397
Human_5	X64994	Zeb_2	U43295
		Zeb_3	U42396

Mudpuppy *Necturus maculosus*

MP-A	AF019237
MP-B	AF019238
MP-C	AF019239
MP-D	AF019240
MP-E	AF019241
MP-F	AF019242
MP-G	AF019243
MP-H	AF019244
MP-I	AF019245
MP-J	AF019246
MP-K	AF019247

(1 cycle). 94°C for 30 s, 55°C for 1 min, 72°C for 40 s (38 cycles), and a final extension at 72°C for 5 min. PCR bands of the expected size (500–540 bp) were excised from the gel with a GeneClean kit (Bio101) and cloned into the pGEM-T vector (Promega). Sequencing was performed with a LiCor automated DNA sequencer. Putative olfactory receptors identified through BLAST searches of GenBank were completely sequenced on both strands. The sequences have been deposited in GenBank with the following accession numbers: AF019237–AF019247.

For phylogenetic analysis, forty representative amphibian, mammalian, avian, and fish olfactory receptor genes were converted to amino acid sequence and aligned with the GCG PileUp program. Due to the large size of the olfactory receptor gene database (>300 genes), only the most diverse representatives from each group, as measured by sequence dissimilarity, were selected. Regions of the dataset where the alignment was obscure were excluded from further analysis.

Phylogenetic trees derived from comparisons of the aligned olfactory receptor genes were constructed by means of distance matrix, neighbor-joining methodologies (with the PHYLIP

package), and with parsimony (heuristic searches using the program PAUP, version 3.1.1). Distance matrix analysis of 100 bootstrapped datasets was conducted to estimate confidence levels of internal branches.

Of the 40 recombinant clones sequenced from the mudpuppy, 11 were found to code for olfactory receptors. Overall, mudpuppy sequences exhibit from 27 to 58% identity to each other at the amino acid level, and 24 to 49% identity with representative rat sequences. *In situ* hybridization shows that each of these receptors is expressed in a small number of olfactory neurons randomly distributed in mudpuppy olfactory epithelium (Q. Zhou, unpub. results).

An unrooted neighbor-joining phylogenetic tree derived from a PAM-1 corrected distance matrix of 132 aligned amino acids (the full length of the gene is ~300 amino acids) is presented in Figure 1. A heuristic parsimony analysis gave rise to a slightly different tree topology (data not shown). Neighbor-joining bootstrap percentages greater than 80% (from 100 bootstrap replicates) are shown on the tree. Regardless of the methods used, all fish representatives are consistently part of a distinct group that includes some amphibian sequences (Xenopus Y08347 and Y08435 and mudpuppy MP-A).

We have cloned 11 olfactory receptor genes from mudpuppy. Phylogenetic analysis of olfactory receptor genes from major vertebrate groups suggests a distinct separation of fish se-

quences and mammalian sequences, while amphibian olfactory receptor genes, both mudpuppy and *Xenopus*, apparently belong to both groups. This finding is surprising because aquatic and terrestrial animals have access to structurally very different odors. That fish-type receptors detect only water-borne odors, whereas the mammalian-type receptors detect only hydrophobic, air-borne odors has recently gained support (2). Mudpuppies, however, are permanently aquatic, so the odors they experience should be the same as fish, that is water-borne, hydrophilic odors. Thus, the expression of mammalian-type olfactory receptors in mudpuppy indicates that these receptors may serve some role other than the detection of volatile odors. The branches separating internal nodes are comparatively short, suggesting a rapid radiation of olfactory receptor genes. A phylogenetic understanding of gene orthology, and hence an understanding of olfactory receptor evolution, requires the acquisition of additional sequences.

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Genes Coding for Reverse Transcriptase, DNA-Directed RNA Polymerase, and Chitin Synthase From the Microsporidian *Spraguea lophii*

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Microsporidia are amitochondriate, unicellular protists that parasitize an extraordinarily broad range of hosts, including immunocompromised humans and many commercially important insect and vertebrate species. In spite of their economic and medical impact, fundamental knowledge of microsporidia biology, and in particular molecular biology, is lacking. Much of our ignorance is due to the absence of axenic cultures; microsporidia only divide within host cytoplasm. To understand the biology of microsporidia better, we have begun a genomic analysis of *Spraguea lophii*, a microsporidian that infects goosefish (*Lophius americanus*), an important commercial finfish.

Phylogenetic analyses based upon comparisons of 18S ribosomal RNA molecules (1), as well as elongation factors (2), identify microsporidia as one of the deepest branching lineages of the eukaryotic line of descent. These results suggest that microsporidian biology contains clues about the origin of the eukaryotic state. Microsporidia contain the smallest nuclear genomes of any known eukaryote (3). The genome size of *S. lophii* is a mere 6.2 M bp. The density of genes within the *S.*

lophii genome must be correspondingly high, and hence a 'shotgun genomic' analysis should yield biologically interesting and phylogenetically useful information.

Tumors containing *Spraguea lophii* spores were harvested from fresh *Lophius americanus* nervous tissue. The spores were released by mincing the tissue with a sterile razor blade and then washed with ice-cold STE (0.1 M NaCl, 10 mM Tris, pH 8.0, 1.0 mM EDTA). The spores were further purified on a 100%, 75%, 50%, 25% Percoll step gradient. The spore wall was disrupted by grinding with a mortar and pestle in liquid nitrogen. This material was then resuspended in 1.5 ml ice-cold TE (10 mM Tris, pH 7.5, 1 mM EDTA) and extracted with Tris-buffered phenol and CHCl₃. Total nucleic acids were precipitated with 2.5 volumes EtOH and 1/10 volumes 3 M NaOAc, pH 5.2.

Primers complementary to hypervariable regions of 18S rRNA coding regions from *S. lophii* and *L. americanus* were used in PCR (polymerase chain reaction) assays to verify the absence of contaminating *L. americanus* genomic DNA. DNA

for a *S. lophii* genomic library was prepared as follows: The >2 kb fraction of a complete Eco-RI digest of 10 µg of genomic DNA was isolated from a 1% agarose gel with a Gene-Clean II kit. The DNA fragments were cloned into Eco-RI digested Bluescript plasmid, transformed into competent *E. coli*, and plated on ampicillin-containing LB agar. Fifty recombinant plasmids were prepared for sequencing with Qiagen columns according to the manufacturer's directions.

One hundred single-pass *S. lophii* DNA sequences were generated from the 50 recombinant clones by sequencing from both directions with LiCor automated DNA sequencing machines. The size of the inserts (>2 kb) precluded overlap of the reads. The average length of read was about 750 base pairs. Sequences were compared to public DNA and protein databases using BLAST search methodologies (4). The sequences have been deposited to GenBank with the following accession numbers: RNA polymerase II AFO19227, chitin synthase AFO19228, and reverse transcriptase AFO19229.

The intracellular parasitic life style of *S. lophii* enhanced the potential for contamination from host DNA. These concerns led us to confirm the source of the cloned DNA by genomic Southern blotting with the insert, or by PCR with primers complementary to the cloned DNA.

BLAST analyses of the 50 *S. lophii* clones have revealed more than 45 sequences with high levels of sequence similarity (BLAST probabilities < 1.0×10^{-13}) to published gene and protein sequences. Among the genes confirmed derived from *S. lophii* are a reverse transcriptase associated with *mariner*-like transposons, chitin synthase, and DNA-dependent RNA polymerase (RNA pol II). As might be expected for an organism with such a small genome, the gene density of *S. lophii* is unusually high. Almost every clone contained a recognizable protein coding sequence. The A + T content of the sequences was 72%. Intergenic regions of the genome are especially A + T-rich, with long uninterrupted stretches of As and Ts. The result is a G + C content that is disproportionate in protein coding regions. This skew should allow for the preferential cloning of protein coding sequences using restriction enzymes that cut G + C rich recognition sites. Preliminary analysis suggests the absence of introns. We will identify conserved upstream control sequences, such as consensus promoters.

The presence of chitin synthase in *S. lophii* lends superficial credence to comparative molecular analyses of alpha tubulins suggesting microsporidia are closely related to fungi (5). We regard this conclusion as premature. Though chitin is widespread among eukaryotes, chitin synthase sequences are as yet poorly represented in molecular databases. The chitin synthase of *S. lophii*, though most similar to a sequence from the ascomycete *Emmericella nidulans*, is nevertheless substantially divergent from fungal chitin synthase sequences. A rigorous phylogenetic assessment awaits the assembly of an evolutionarily diverse chitin synthase database. The presence of chitin synthase suggests that the tough spore cell wall of microsporidia contains chitin.

The discovery of a reverse transcriptase most commonly associated with retrotransposons was unexpected. The *S. lophii* sequence shares high levels of similarity to a large number of mobile genetic elements (*e.g.*, gypsy and TED) described in dipteran insects as well as a reverse transcriptase isolated from the tunicate *Ciona*. The data suggest that genetically similar transposable elements are mobile across the broadest spectrum of eukaryotes.

Our clone of DNA-dependent RNA polymerase contains only the 5' end of the coding sequence (approximately 180 bp). Work is in progress to obtain a full length sequence of the gene for further phylogenetic analysis.

This work was supported by grants from the National Institutes of Health and the G. Unger Vetlesen Foundation.

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Apparatus for Measuring Steady-state ATP Utilization Rates of Single Muscle Fibers

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The two major ATP-requiring processes during muscle contraction are force generation by crossbridges and pumping of Ca^{2+} by the SR. Measurements of the ATP used by muscle fibers are crucial (1) for partitioning the ATP usage between

crossbridges and Ca^{2+} pumps, (2) as a probe of crossbridge kinetics, and (3) as a measure of the overall energetic cost of contraction which can be related to the energetics of behaviorally important activities such as locomotion and sound production. Here we describe an apparatus for simultaneously measuring force production and the steady-state ATP utilization rate of "skinned" muscle fibers, a device that is not limited by

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diffusion of substrates, as are some conventional fluorometric ATPase apparatuses. "Skinned" muscle fibers, one in which the surface membrane is permeabilized or removed, are used so that the interior of the muscle cell can be exposed to various substrates and enzymes required for the ATPase measurement.

The force-generating crossbridges in muscle are powered by the hydrolysis of ATP. In intact fibers the ADP produced is regenerated to ATP by creatine phosphate (CP), catalyzed by creatine phosphokinase (CPK). To measure the rate of ATP hydrolysis in our apparatus, "skinned" fibers are used so that the CP regenerating step can be bypassed, and ATP hydrolysis can be coupled to a glycolysis-like cascade that takes advantage of the fluorescent properties of NADH. Instead of containing CP (and CPK) to regenerate ATP used by the fiber, phosphoenolpyruvate (PEP) is used in our solutions. The reaction is catalyzed by pyruvate kinase (PK):



This reaction is then coupled to the oxidation of NADH to NAD, and the reduction of pyruvate to lactate, catalyzed by lactic dehydrogenase (LDH):

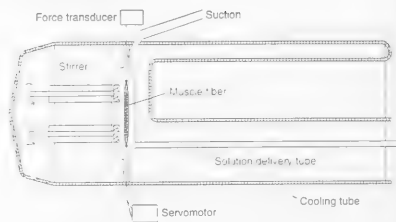


In the net reaction, one mole of ADP and NADH are used as one mole of ATP and NAD are produced. NADH fluoresces upon excitation at ~320–400 nm, whereas NAD does not. Because of the stoichiometric relationship between ADP production and NADH oxidation, ATP use by the muscle (production of ADP) can be measured by the decrease in NADH fluorescence of the solution surrounding the muscle fiber.

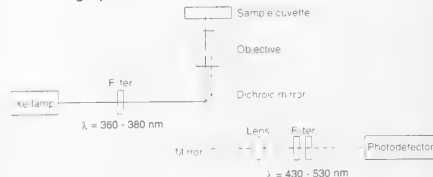
This assay has been employed on several types of muscle fibers under various conditions (1–6). Control experiments have previously shown that the decrease in fluorescence (ATP turnover) is independent of PK, PEP, and LDH concentrations over a wide range of values in some fibers types (1, 2). However, in an unstirred chamber the NADH concentration is critical. If [NADH] is too high (larger than 2 mM), the absorption of the exciting light is high, and only a thin layer of the solution will be penetrated. If [NADH] is too low, the NADH within the fiber (and near to it) is quickly converted into NAD, a gradient is set up, and the results will be misleading. Also in an unstirred chamber, the assay depends on obtaining an average sample from the entire volume of solution within the cuvette (*i.e.*, integrating over the NADH gradient, which is impossible to reproduce for calibration purposes). Therefore to obtain accurate, quantitative ATPase rates, a relatively low [NADH] and a well-stirred chamber must be used.

Although some previously designed chambers provide some circulation of the solution by means of miniature diaphragm pumps (2, 3), the one discussed below provides very rapid mixing probably crucial in fibers with very high ATP utilization rates. The ATPase apparatus described incorporates the optics for measuring the fluorescence change in a cuvette in which the muscle fiber is positioned and solutions containing the appropriate enzymes and substrates for the coupled assay can be rapidly exchanged (Fig. 1A, B). An excitation beam (360–380 nm) is focused in the cuvette, and the emission (430–

A. Muscle cuvette chamber



B. Fluorescence light path



C. Swimbladder ATPase

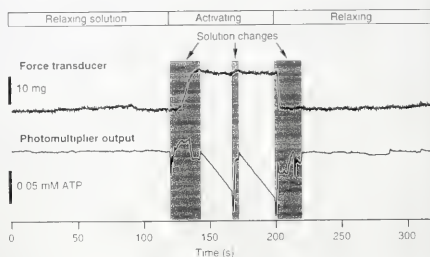


Figure 1. An apparatus for measuring the ATPase rate of single muscle fibers. (A) The chamber consists of an anodized aluminum block with a 12 μ l solution well. Slots cut at the ends of the well allow pins attached to a force transducer and servomotor to enter the well; the muscle fiber is "T-clipped" onto the tips of these pins. Glass coverslips (not shown) cover the top and bottom of the well and form a "cuvette" of fixed volume. Solutions enter the well at one end via a stainless steel tube, pass through the well and, at the opposite end, are removed via a suction tube placed at the surface of the well. Used solution is removed at exactly the same rate that new solution is introduced. The solutions are held in plastic reservoirs, and solenoid valves, regulated by a digital computer signal, control the flow. Two micromachined stirring propellers, placed along one side of the well, are attached via stainless steel shafts to gear-reduced DC motors. (B) The chamber is mounted on a Leica DM-IRB inverted microscope fitted with epi-fluorescent illumination. During ATPase measurements, near UV light produced by a Xe lamp is focused into the sample cuvette by the objective lens, causing the NADH to fluoresce. The emission light (dashed line) passes back down through the lens and dichroic mirror before detection by a solid state photodetector. (C) While in relaxing solution, fluorescence changes little. Upon influx of activating solution, the fiber generates force and uses ATP at a high and constant rate, as signified by the steep decline in fluorescence. To prevent [NADH] from dropping too far during steady activation, the chamber is then flushed with fresh activating solution. The cuvette is finally flushed with relaxing solution which causes the fiber to relax, and ATP use to decline.

530 nm from NADH) is measured with a solid state photodetector. To ensure vigorous stirring, our apparatus incorporates two micro-machined propellers. The blades are attached to stainless steel shafts and sealed into the chamber with miniature "o" rings and Kel-F bushings. During ATPase measurements, the muscle is maximally activated, and the fluorescence signal is monitored. Because the cuvette is well stirred, the NADH fluorescence can be sampled anywhere within it except for the small volume occupied by the fiber itself.

Fibers are skinned by exposure to 3% Triton-100. The size of the preparation is adjusted according to its ATPase to provide a measurable signal without diffusion limitation. Thus for fibers of low ATPase, moderate sized (60–80 μm) single fibers are used. For fibers with high ATPase, the diameter is reduced to 40–50 μm . Depending on the fiber type this may involve longitudinal splitting of large diameter fibers or the use of small bundles of very small diameter fibers.

We used solutions (ionic strength = ~ 200 mM) of pCa 8 (relaxing) and 4.3 (activating) both containing the enzymes (PK 1000 Unit/ml, LDH, 200 Units/ml) that link the hydrolysis of ATP to the oxidation of NADH (concentration 0.5–0.75 mM). P^i , P^i -di(adenosine-5')pentaphosphate is added to the solutions to inhibit myokinase activity (7), and thapsigargin is added to inhibit ATP utilization by SR-Ca^{2+} pumps. Figure 1C shows a sample trace. In relaxing solution, there is little change in fluorescence. Upon activation, force rises and fluorescence declines linearly at a rapid rate, signifying a high and constant rate of ATP use by the fiber. During this steady activation, the

chamber is flushed with fresh activating solution to prevent [NADH] from dropping too far. The chamber is finally flushed with relaxing solution.

The system is calibrated and the kinetics assessed by injecting known amounts of ADP and examining the time course of fluorescence changes. This shows that the reaction goes to completion and that the overall response time of the reaction and the chamber is about 1.5 s. Further, we have found that our measure of ATP utilization rate in the fastest vertebrate muscle (toadfish swimbladder) does not decline even after reducing [NADH], 3-fold, [PK], 10-fold, and [LDH], 2-fold. This suggests that there is no diffusional limitation in the measurement.

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Do Tea Polyphenols Protect Dogfish Lens (*Mustelus canis*) Catalase Against UV Damage?

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The dogfish lens is damaged by UVA (320–400 nm) photo-oxidation. The aim of this research to elucidate this damage and to study its protection by antioxidants (1–3). Indeed, the dogfish lens was adopted as a model because physical damage (*i.e.*, opacity and cytoskeletal breakdown) occurred in response to UVA radiation; catalase activity of the lens is markedly diminished by UVA irradiation at levels that the lenses of land animals could receive from sunlight. But dogfish lens catalase activity can also be protected against UVA exposure by antioxidants: alpha-tocopherol and deferoxamine were the most effective protectors. The experiments reported here were done to determine whether the polyphenols extracted from tea, which are known to be antioxidants, would also retard such lens photo-damage.

Fresh dogfish lenses (*Mustelus canis*) were obtained according to procedures approved by the Animal Use Committee at the Marine Biological Laboratory. They were placed in elasmobranch Ringer solutions for incubation procedures. In some cases, polyphenols were added to the solution at 50 $\mu\text{g}/\text{ml}$; these

were epigallocatechin gallate (EGCG) or purified tea polyphenols (Lipton Tea Company). The solutions were incubated in the dark or exposed to UVA from a Woods lamp for 12 h, at 2.5 mW/cm^2 , at $\sim 20^\circ\text{C}$, and under an atmosphere of 95% air; 5% CO_2 . After the incubation, the lenses were washed with elasmobranch Ringer solutions, and O_2 production from 0.88 mM H_2O_2 (*i.e.*, catalase activity) by each lens was measured with an O_2 electrode and meter (Microelectrodes, Inc.) and recorded against time with a chart recorder (1, 2). The $\% \text{O}_2$ produced per minute was compared with that produced by Sigma $2\times$ crystallized beef liver catalase (84 $\mu\text{g}/\text{ml}$ or 36 units/ml).

Table I shows that UVA treated lens O_2 production (*i.e.*, catalase activity) was inhibited by 57% as compared with the dark control lenses. The UVA treated lens catalase activity was protected by 73% when 50 $\mu\text{g}/\text{ml}$ of EGCG was present.

Table I also shows that GTP (green tea polyphenols—purified) protects catalase activity appreciably (by 94%). BTP (brown tea polyphenols—purified) was also active (85%), but less so than GTP. Table I shows further that Sigma $2\times$ crystal-

Table 1

Effects of polyphenols on the inhibition of dogfish lens catalase activity by UVA irradiation

Sample category*	Oxygen production per minute: percent of controls	
Whole lenses	Mean of two experiments	
Dark control	100	
Dark plus EGCG ¹	105	
UVA exposed	43	
UVA exposed plus EGCG	73	
UVA exposed plus GTP ²	94	
UVA exposed BTP ³	85	
Extracts	Lens capsule epithelium homogenized supernatant	Sigma 2× crystallized beef liver catalase
Dark controls	100	100
Dark plus EGCG	—	89
UVA exposed	10	16
UVA exposed plus EGCG	55	83

* Average of 2 or 3 measurements; variation was $\pm 10\%$.

¹ EGCG—Epigallocatechin gallate.

² GTP—Green tea purified polyphenol extract.

³ BTP—Brown tea polyphenol extract.

lized beef liver catalase itself was similarly protected from UVA inactivation by EGCG.

To determine whether the polyphenols entered the lens, we soaked several dogfish lenses in 100 $\mu\text{g}/\text{ml}$ of GTP or EGCG

for 12 h. The brownish-pink color of the polyphenol solutions stained the lenses, but when the stained capsule epithelium was removed, no color remained in the lens cortex. Homogenization of lens capsule epithelium and centrifugation at $10,000 \times g$ indicated that the stain was present in both supernatant and sediment. Spectral measurements of lens capsule epithelial cell homogenates also showed that the polyphenols enter the lens capsule epithelial cells as they retained the absorbance of the polyphenols. Fluorescence measurements of extracts also confirm the spectral absorbance data in that the extracts retained the fluorescence of polyphenols.

In conclusion, we have found that EGCG, GTP, and BTP do retard the loss of catalase activity from dogfish lens epithelial cells that have been exposed to UVA. Earlier research showed that the damaging effects of UVA on lens epithelial cells is due to excitation of molecular species leading to the formation of singlet excited-state and hydroxyl radicals (4). Thus, we speculate that tea polyphenols can protect the lens against the action of such excited-state species.

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Do Alpha-Crystallins Protect Catalase Against UV Damage?

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The original finding that lens α -crystallin could function as a molecular chaperone, protecting many proteins against heat denaturation, was made by Horwitz (1). Not only did α -crystallin protect structural proteins of the lens, but it also protected selected enzyme activities against heat-induced inactivation. That UVA irradiation causes structural and functional damage to the catalase of the lens was discovered by Zigman *et al.* (2). In this preliminary study, we tested the ability of bovine α -crystallin to protect the activity of catalase from UVA inhibition.

Sigma 2× crystallized beef liver catalase (0.84 mg/ml; 36 units/ml) in 0.1 M, pH 7.2 phosphate buffer was exposed to

Woods lamp UVA radiation at $\sim 2.5 \text{ mW}/\text{cm}^2$, for 12 h at 20°C , under an atmosphere of 95% air:5% CO_2 . The optical densities at 280 nm (O.D._{280}) of both catalase and α -crystallin solutions were determined spectrophotometrically. The O.D._{280} of both solutions ranged from 0.350 to 0.700 units. Mixtures of catalase and α -crystallin solutions having an equivalent O.D._{280} , or in which the α -crystallin O.D._{280} was twice (molar equivalence) or three times that of the catalase, were prepared and irradiated. One variation of the above protocol was to preincubate catalase for 12 h with a solution of UVA-irradiated tryptophan (UV-trp; 1% in PO_4 buffer) as a catalase inhibitor. This was done to assess the ability of α -crystallin to protect catalase activity from UV-trp. Another variation was to use a $10,000 \times g$ supernatant of dogfish lens-capsule epithelium as the catalase source. Its preparation is described in our companion short report (3).

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Table I

Effects of α -crystallin on the inhibition of catalase activity by UVA irradiation

Category		Oxygen production per minute: percent of controls
Effect of UVA on purified catalase activity	Dark control	100 $\pm$ 10*
	UV exposed	45 $\pm$ 8
	UV exposed with α -crystallin (a)	48 $\pm$ 4
	UV exposed with α -crystallin (b)	87 $\pm$ 7
	UV exposed with α -crystallin (c)	87 $\pm$ 8
Effect on UV-trp on purified catalase activity	Dark controls	100 $\pm$ 7
	Dark plus UV-trp preincubation	50 $\pm$ 5
	Dark plus UV-trp α -crystallin (b)	84 $\pm$ 6
Effects of UVA on dogfish lens-capsule epithelial-cell catalase	Dark control	100 $\pm$ 4
	UV exposed	10 $\pm$ 1
	UV exposed with α -crystallin added	33 $\pm$ 2

* Refers to average of results of two experiments that varied by $\pm 10\%$.

¹ Refers to Sigma 2 $\times$ crystallized bovine liver catalase.

² Refers to α -crystallin added to catalase at equal O.D.₂₈₀.

³ Refers to two times the O.D.₂₈₀ of catalase.

⁴ Refers to three times the O.D.₂₈₀ of catalase.

Alpha-crystallin was added during UVA exposure to reduce the damaging effect on lens catalase.

Oxygen production (*i.e.*, catalase activity) was measured with an O₂ electrode and meter (Microelectrodes, Inc.) and displayed as a function of time with a chart recorder. The percent O₂ produced per minute for treated catalase was compared with the activity of known catalase standards.

Table I shows that α -crystallin partially protects purified catalase activity when present at 2 $\times$ (equimolar) and 3 $\times$ the O.D.₂₈₀ of the catalase. When UVA-exposed 1% tryptophan (UV-trp) was added as the perturbant of catalase activity (4), the results in Table I were also obtained: the presence of 2 $\times$ O.D.₂₈₀ (or equimolar) α -crystallin protected the catalase activity from UV-trp by about 34%. When homogenized and centrifuged dogfish lens-capsule epithelial-cell catalase activity was used to test α -crystallin's protection against UVA (Table I), α -crystallin was also found to be protective.

In the living lens, most catalase activity is in the epithelium where α -crystallin is also present. A higher concentration of alpha is present in the soluble phase, while catalase is mainly in the peroxisomes. However, cellular damage releases catalase into the soluble phase.

The results show that α -crystallin can protect against the UVA inhibition of catalase enzymatic activity, as was shown for other enzymes by Borkman *et al.* (5). The concept that α -crystallin has a great variety of molecular chaperone activities is supported by this study.

We dedicate this to the fond memory of Arlene Horwitz, who appreciated the time she spent at Woods Hole and the Marine Biological Laboratory. We thank Linlin Ding for providing α -crystallin (Jules Stein Eye Institute) and Ray Borkman and Grady Knight (Georgia Institute of Technology) for providing the molar extinction coefficient for α -crystallin. Supported by: National Eye Institute (NIH) # EY 3897 (Horwitz).

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Reference: *Biol. Bull.* 193: 255-257. (October, 1997)

Pulsatile Movement of *Hermisenda*

Herman T. Epstein (Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

Hermisenda contracts when it is shaken, reducing the length of its foot. Experiments by myself and others on conditioned responses of *Hermisenda* show that 60 to 80% of control animals (living in normal seawater) contracted in response to light alone after several days of training with light coupled with shaking (1, 2, 3). When lead acetate (PbAc) is added to seawater, the percentage of animals contracting drops in a dose-related manner to values ranging from 40% to 10%. During this work, conditioning never reached 100 percent.

An explanation for this may be that these nudibranchs move

in a pulsatile way. This means that animals that happen to be in an extension phase do contract when light appears, but the resultant foot length shows essentially no change. The pulsatile movement of *Hermisenda* apparently has not been previously reported.

Animals were kept in an incubator at 10°C in a 12-h on/off cycle of a fairly dim orange light. They were trained and tested in a flat plastic holder with 16 parallel lanes. For training, we exposed animals to 6 s of intense white light. The shaking started 2 s after the light was turned on and continued until the

end of the 6-s period. This was repeated at 1-min intervals 30 times daily on 3 successive days. On the fourth day, the animals were tested with light alone.

We videotaped the movements of the animals for 1 min in the orange light, measured the lengths of the animals at 1-s intervals and computed the length differences between the start and end of successive 3-s intervals. Lengths were measured on the monitor to an accuracy of about 0.5 mm; this agrees with the value of 0.24 mm for the standard error in the sense that two standard errors, or 0.48 mm, include almost all the actual data. Thus changes larger than 0.48 mm (so that $t \geq 2$; $p < 0.03$) are truly changes and not just measurement variations.

Typical results (Fig. 1, top) show many periods of increase and decrease in length for six animals living in aerated natural seawater. Multiples of 3 mm were added to the data for the various animals to separate the curves from each other.

Figure 1 (middle) presents the results for five animals living in aerated natural seawater containing 1 ppm of lead acetate. This figure also shows periods of increasing and decreasing length. The range of values differs from that in the top figure because different multiples of 3 mm were added.

The test data were then studied to determine whether a sufficient number of animals actually manifested a length extension so as to account for the missing 30% of contractors.

For eight control animals, of 132 length differences measured, 46 were extensions equal to or greater than the criterion of two standard errors, or about 0.5 mm; this 34.8% is close enough to the observed missing 30% contraction rate to explain the apparent failure of that percentage of the animals to manifest contractions. This suggests that close to 100% of the animals were successfully trained.

When previous data for another group of 23 control animals were studied, 43.4% showed extensions during exposure to orange light. The standard errors were such that the value was statistically equivalent to the 34.8% described above.

A similar analysis of the movement of 24 animals living in 1 ppm PbAc gave similar pulsatile results. Of 292 length differences measured, 113 were extensions equal to or greater than 0.5 mm; this 38.7% does not differ significantly from the value found for control animals.

Figure 1 (bottom) shows that the percentage of animals attaining the criterion of lengthening at least 0.5 mm reaches an asymptote by 4 s after every starting point. The percentages reached are sufficiently similar to those at 3 s that changing from using the 3 s interval would not make a significant difference in the analysis of this explanation.

Data from previous testing sessions of control animals were used to determine how often an extension was followed by another extension in 37.4% of the situations; by a contraction in 31.3%; and by no change in 31.3% of the situations. For animals living in 1 ppm PbAc, 34.1% of extensions were followed by a contraction. These values show that the non-contracting animals were not just habitual non-contractors.

Thus, the results of this work support the existence of pulsatile movements in *Hermisenda*. Moreover, the percentage of animals extending during typical 3-s to 4-s intervals, measured during testing, could account quantitatively for the failure to detect learning by all of the animals.

HERMISSENDA

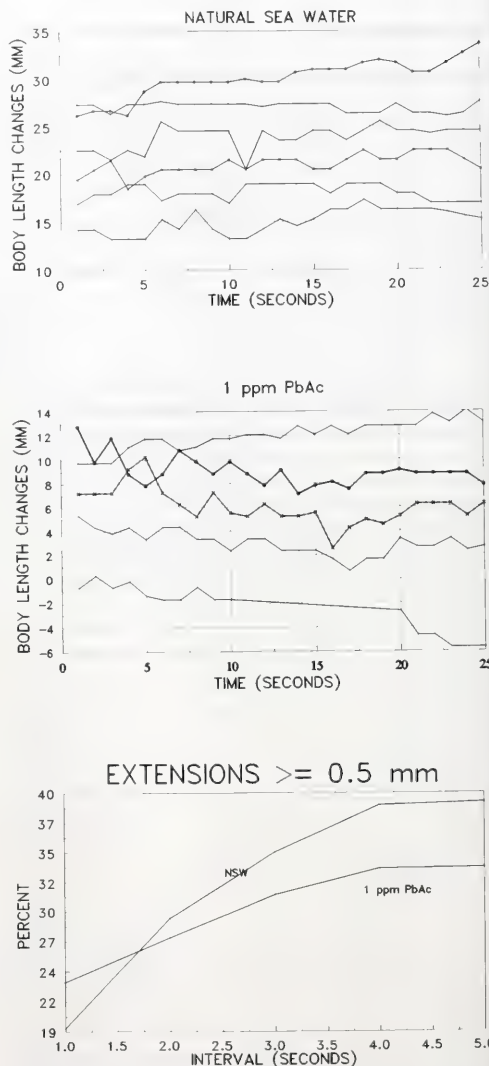


Figure 1. Top: Lengths of six *Hermisenda* individuals living in natural seawater recorded over a 25-s period. After the bottom animal, multiples of 3 mm were added to the values to separate the curves for better visibility. Middle: As for the top figure except that the five animals were living in seawater containing 1 ppm of lead acetate. Bottom: The percentage of extensions taken from data as in the two figures above in which the lengths exceeded two standard errors as measured for intervals of 1 to 5 s.

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Development of the Motor System in the Limbs of Larval Lobsters (*Homarus americanus*)

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Larvae of clawed lobsters (*Homarus* sp.) are released from eggs attached to the pleopods on the abdomen of the female

(1). There are three pelagic larval stages and a fourth pelagic post-larval stage. In the first three stages, the shape and disposition of the body differs from that of the adult. Their thoracic appendages are biramous, and the outer branches or exopodites are equipped with a fan of setae that acts as a paddle; thus, when the exopodites beat rhythmically they provide locomotory power (Fig. 1A). When the animal molts to the fourth stage, it remains pelagic but assumes the adult body form. The exopodites of the thoracic limbs are lost, and locomotion is provided by the endopodites—which become the pereopods, or walking legs—and by the biramous pleopods of the abdomen. There are some innervated muscle fibers in the endopodites of the larvae of *H. americanus* (2) and, in the case of the chelipeds (which the animals use to manipulate food), they are functional even in Stage I (3). In *H. gammarus*, movement of the endopodites of the walking legs is limited to slight twitching movements, which have been ascribed to mechanical connection of the two rami through the basis, rather than to any active movement of the endopodite itself (4). This report describes aspects of the relationship between the two locomotory systems during the development of the first four stages of the American lobster, *Homarus americanus*.

Animals were obtained from the Lobster Rearing Facility at the New England Aquarium, kept in aerated, running seawater at 8°C and fed *Artemia*. Electrical recordings from the muscles were made with two conventional intracellular recording systems. The microelectrodes were filled with 2 M KCl, or 1 M LiCl tip-filled with Lucifer yellow dye (Sigma, St. Louis, MO). The motor neurons were filled by retrograde infusion of a saturated solution of Lucifer yellow, 1.5% CoCl₂, or both, and cell counts were made by viewing the soma following fluorescent excitation or deposition of intracellular CoS.

Larvae of Stages I–III were pinned to a Sylgard-lined dish

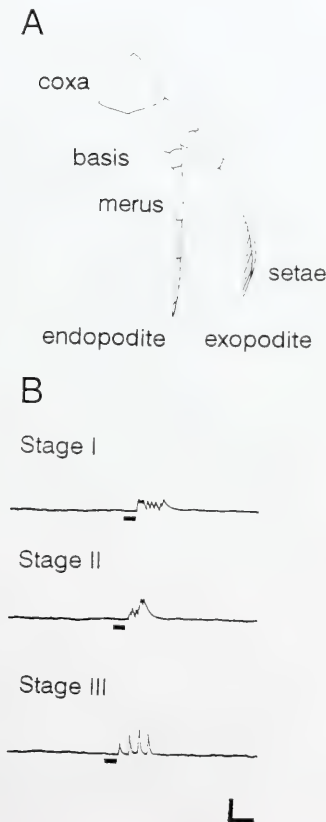


Figure 1. (A) Diagram showing the arrangement of the elements of the biramous thoracic appendages of the Stage I–III larvae of the lobster *Homarus americanus*. (B) Examples of reflexly evoked activity in the muscle fibers of the meropodite muscles in larval stages I–III showing that some endopodite motor neurons have functional central connections in all larval stages. Bar indicates application of touch stimulus. Time scale, 200 ms; amplitude, 10 mV.

Table 1

*Numbers of somata counted in the ganglion following dye infusion at the distal end of the basis in animals of different stages**

	Stage I	Stage II	Stage III	Post larva and adult
Exopodite	5, 5	6, 5	5, 6, 5	
Endopodite	13, 13, 14	15, 12	12, 13, 13, 14, 15	12, 13, 13, 13, 14

* These numbers demonstrate that something approaching the full complement of motor neurons is present at hatching, but they cannot be considered definitive until silver intensification and antibody staining has been completed.

and their limbs immobilized with petroleum jelly. Simultaneous recordings were made from the basal muscles producing the rhythmic beating of the exopodites and the muscle bundles developing in the merus of the endopodite of the first walking leg. In no case was there any apparent temporal relationship between bursts of activity in the basal muscles of the exopodite and the EPSPs recorded in the muscles of the merus, although endopodite segments were occasionally seen to twitch at a frequency similar to that of the exopodite contractions in some preparations. The endopodites of the first walking legs are functionally innervated at hatching, because light touches with a fine probe to the hairs at the tip of the endopodite evoked reflex activity in muscle fibers of the merus in all three larval stages (Fig. 1B). Reflex activity was also observed in other endopodite muscles. Similar touches to the ends of the exopodite did not produce reflex activity in the endopodite muscles or start the beating rhythm in quiescent animals.

Intracellular dye was infused through the cut ends of the exopodite muscles so that the innervating motor neurons also took up the dye. This method will not reliably fill every neuron in every preparation, but observation of multiple preparations permits an estimate of the motor neuron complement (5, 6). Five or six larger motor neurons were seen to be associated with the basal exopodite muscles ($n = 7$; see Table I). Several smaller neurons were stained inconsistently and could not be resolved clearly. Similarly, infusion of intracellular dye into the end of the main endopodite nerve, as it passes distally out of

the basis, revealed 12–15 motor neurons in all four stages, as well as in the adult animal ($n = 15$; see Table I). Further details of these neurons and more precise cell counts may be possible after silver intensification or treatment with an antibody to Lucifer yellow.

Although the cell counts are only approximate, they indicate that about the full complement of motor axons is present in the endopodites of the larvae (7); but these neurons do not appear to be connected centrally to the rhythmic generators responsible for the locomotory rhythm. Some of the neurons receive input from the developing sensory system of the endopodites and are involved in withdrawal reflexes. The experiments do not, however, reveal how many of the axons present make functional connections with the endopodite muscles, and this may change with each stage. No reflex withdrawal activity was seen in response to touches applied to the exopodite, suggesting that there may be no connection between exopodite and endopodite sensory systems. The lack of any withdrawal reflexes in the exopodite suggests that the exopodites lack sensory innervation, but this requires further investigation.

I gratefully acknowledge all manner of assistance from Dr. Rainer Voigt, without which this project would not have been possible. My thanks also to Drs. Arthur Humes and Ken Foreman for the loan of equipment, to Dr. C. K. Govind for advice on larval development, and to Drs. E. A. Kravitz and T. J. Trott for assisting me to obtain lobster larvae. The larvae used in the project were supplied by the New England Aquarium Lobster Rearing Facility, Boston, Massachusetts, which is supported by NIH, NSF and the Human Frontiers Project. I thank the staff there for their cooperation. The work was supported by a grant from the Australian Research Council to DLM at the Department of Zoology, University of Melbourne, Australia.

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Neurons Associated With a Novel Motor Pattern Expressed During Metamorphosis of the Hawkmoth, *Manduca sexta*

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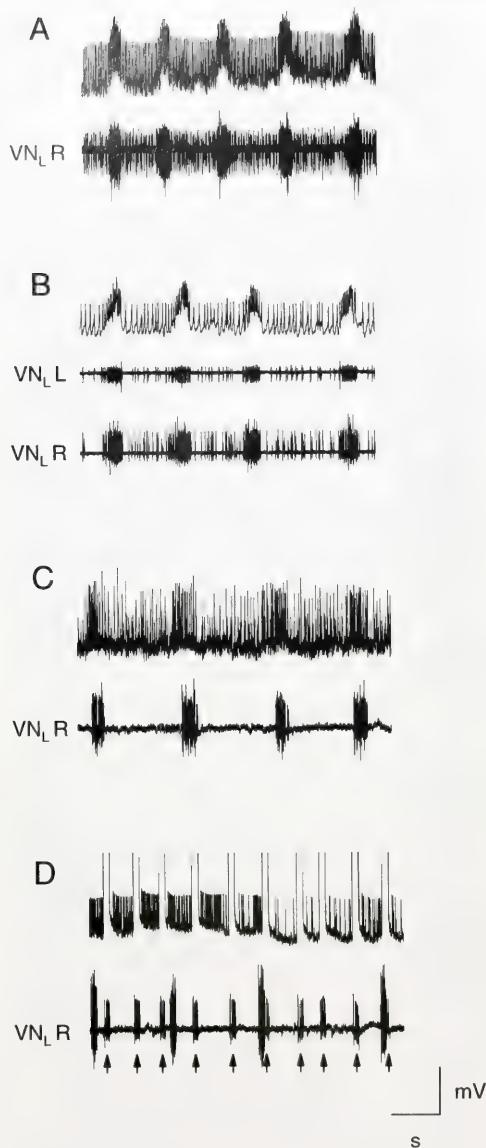
Metamorphosis of the hawkmoth, *Manduca sexta*, involves radical changes in behavior that are mediated by reconfiguration of existing body tissues, including muscles and neurons. Reconfiguration can take the form of muscle degeneration and formation, neuronal death and neurogenesis, and neuronal regression and expansion (1). The consequences of these changes are reflected in motor patterns that have been investigated in pre-ecdisis (2) and ecdisis (3). A recently described motor pattern in the pupa (4, 5) is characterized by rhythmic activity in the larval accessory planta retractor muscles (APRMs) that is synchronous between abdominal segments 2 (A2) and 3 (A3). In the larva, APRMs in segments A3 to A6 retract the prolegs and are thus locomotory. In the pupa, which lacks prolegs, the APRMs in A4 to A6 have degenerated while those that remain, in segments A2 and A3, function to circulate hemolymph in the developing wings (6). The pattern of activity of these muscles is established, stabilized, and terminated within a 2-week period around the time of pupation. This is the most dramatic functional respecification of the neuromuscular system known in this species, but the component neurons that produce the pattern have yet to be described. As a necessary first step to describing the circuitry that produces the pupal motor pattern, I examined the activity of neurons within A3 during expression of the pattern.

Larval *M. sexta* were obtained from a colony maintained at the University of Oregon, and reared through pupation at the Marine Biological Laboratory, Woods Hole, Massachusetts. Animals were selected either 2 or 3 days before pupation (prepupae), or 2 days after pupation, because the pupal motor pattern is established and stabilized, respectively, at these times (4). Morphological characteristics of the larva were used to estimate the time before pupation. Animals were cold-anesthetized before each experiment. A dorsal incision exposed the body cavity, and the gut was removed. The central nervous system, either from the supraesophageal ganglion to A5 or from A1 to A5, was removed and pinned out, ventral side up, onto a dish lined with Sylgard (Dow Corning, Midland, MI). The right and left ventral lateral nerves (VN_L) of ganglia A3 and A4 were left intact. This nerve contains motor axons that innervate ipsilateral proleg muscles of the same segment. Often, the attached APRMs were also removed. Ganglion A3 was desheathed with fine forceps (7) and then examined under a dissecting microscope with a substage dark-field illuminator which enabled the location of neuronal cell bodies. Stimulation electrodes were placed onto the right and left VN_L s in A3 and A4 and were used to record proleg motoneuron activity. Borosilicate microelectrodes, pulled to a resistance of about 40 M Ω , filled at the tip with 5% Neurobiotin (Vector Laboratories, Inc., Burlingame, CA), and backfilled with 2 M KAc, were used for intracellular recording from neuronal cell bodies. Neurobiotin was iontophoretically injected into the cell using 4–10 nA of hyperpolarizing current. Filled cells were processed with Avidin conjugated to Lucifer

yellow and examined with a Zeiss Axioplan 2 compound microscope equipped with an ultraviolet light source filtered with a FITC filter set (excitation wavelength 450–490 nm).

Rhythmical bursting that was similar to the previously described pupal motor pattern (4) was recorded from prepupal and early pupal preparations. The pattern recorded from prepupae was typically less regular and the bouts were of shorter duration (about 2–5 min) than those recorded from early pupae (about 1 h), which is in agreement with previous findings from prepupal preparations (4). Recordings from one motoneuron in A3 of an early pupa showed bursting in phase with the pattern (Fig. 1A). The position of the cell body within the ganglion and the presence of time-aligned spikes in the intracellular and extracellular records suggest that this cell is the accessory planta retractor motoneuron (APR) that innervates APRM. Another neuron, in a prepupa, was also rhythmically active with the motor pattern (Fig. 1B) but had no effect on the activity recorded from VN_L . This neuron is likely a proleg-related motoneuron, as its axon runs out the ventral nerve (VN), and its cell body is located within a dorsal cluster of motoneurons that innervate other proleg muscles via VN_L or the posterior ventral nerve (VN_P). A third, unidentified neuron in a prepupa was also found to be rhythmically active in phase with the motor pattern (Fig. 1C, D). Moreover, injection of about 10 nA of depolarizing current into the cell (arrows in Fig. 1D) induced an increase in the spiking of the cell, as well as spiking in one of the motor units in VN_L , suggesting that the cell is a premotor interneuron. In all three neurons described above, membrane depolarizations underlie the bursting that is in phase with pattern expression. Moreover, injection of brief (~50–100 ms) depolarizing current pulses (between 4 and 10 nA) into these neurons produced a depolarization of the membrane potential that was activated only for the duration of the pulse (data not shown), suggesting that the activity of these neurons during pattern expression is driven, at least in part, by presynaptic input.

The data presented here demonstrate that the motor pattern expressed in the prepupal period is similar to the pupal motor pattern, although it is not as robust as after pupation. This suggests that the circuitry underlying the pupal motor pattern is, at least partially, configured before pupation. This idea is supported by the finding that, in the prepupa, a proleg motoneuron other than APR is active with the rhythm (Fig. 1B), and a putative premotor interneuron (Fig. 1C, D) is also active with the rhythm and induces spiking in a motoneuron that projects out VN_L . Although the lack of a self-maintained depolarization in APR and another unidentified motoneuron does not rule out the existence of endogenous bursting mechanisms in these cells, it does suggest that the rhythm is, in part, synaptically driven by premotor interneurons. These premotor interneurons may, themselves, be driven by descending inputs from the neurons anterior to A1, because no pattern was recorded from preparations that included only the abdominal ganglia (see, also, reference 4).



Further investigations into the identity of the premotor interneurons and the role of descending input from the brain and the thoracic ganglia must be made if we are to have a detailed description of the circuitry that produces the pupal motor pattern. Such a description can provide a framework upon which to investigate the developmental processes that sculpt out circuits for rhythmic motor patterning. This system is an ideal model for examining these processes, since the dramatic functional respecification of the proleg neuromuscular system occurs over a period of a few days. Thus we may further understand the mechanisms by which the nervous system may accommodate adaptational changes that are dictated by the developmentally regulated behavioral demands of the organism.

I wish to thank Dr. J. C. Weeks for supplying larval *Manduca* and necessary equipment, as well as providing comments on a previous version of the manuscript. I also thank Dr. D. J. Sandstrom for useful discussions on the project. Financial support was provided by the Grass Foundation.

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Figure 1. Intracellular recordings (top trace) from neurons active with the pupal motor pattern in *Manduca sexta* (extracellular recordings from VN_L , bottom traces). (A) Recording from APR in an early pupa showing membrane depolarizations underlying the bursts. APR was identified by the position of its cell body within the ganglion (A3) and time-aligned spikes with VN_L . (B) Recording from a proleg-related motoneuron in A3 of a prepupa. Extracellular recordings from right and left VN_L ($VN_L R$ and $VN_L L$, respectively) are shown. Tonic firing of the motoneuron was not correlated with tonic activity in the ipsilateral nerve ($VN_L R$), whereas the large depolarizations that underlie the bursts are in phase with VN_L bursting. (C) Bursting by an unidentified neuron in A3 of a prepupa is in phase with bursting recorded from VN_L . (D) Same cell as in C during rhythmic stimulation with 500-ms depolarizing pulses of about 4 nA (arrows). Stimulation induced spiking in one of the units recorded in VN_L , suggesting that this neuron is presynaptic to the motoneuron. Scale bars: 15 mV in A and B; 4 mV in C and D; 4 s in A; 2.5 s in B and C; 5 s in D.

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Fast Voltage-sensitive Dye Recording of Membrane Potential Changes at Multiple Sites on an Individual Nerve Cell in the Rat Cortical Slice

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Understanding the biophysical properties of single neurons and how they process information is fundamental to understanding how the brain works. But the regional electrical properties of neurons are extraordinarily complex and impossible to understand in the absence of detailed, spatially well-resolved measurements of electrical signals. Recent developments, carried out on invertebrate neurons (1), now permit the monitoring of membrane potential transients at many sites by an optical imaging technique that employs intracellular voltage-sensitive dyes. Here, we report an extension of this same approach to vertebrate neurons. Earlier results of these kinds of measurements have been reported (2).

Experiments were carried out on layer 5 pyramidal neurons

in slices from the neocortex of P14–P18 rats. Individual neurons were selectively stained, by diffusion from a patch pipette of the positively charged, membrane impermeant, voltage-sensitive, fluorescent, amino-naphthyl styryl dye JPW3028. The fluorescent image of the cell was projected onto a 464-element photodiode array. Optical signals associated with action potentials, expressed as fractional changes in fluorescent light intensity ($\Delta F/F$), were about 1% in recordings from neuronal processes. The signal-to-noise ratio was sufficiently large that action potential signals could be monitored from multiple sites on dendritic processes (Fig. 1).

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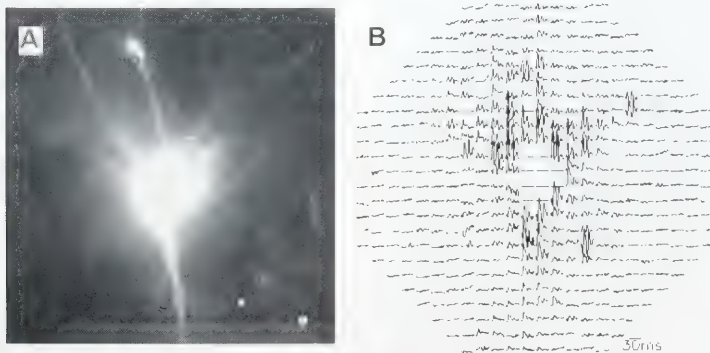


Figure 1. (A) Fluorescent image of a pyramidal neuron. (B) Optical recordings of action potential signals from elements of a photodiode array. Each diode received light from a $14 \times 14 \mu\text{m}$ area in the object plane. Each trace represents the output of one diode, when the neuron was stimulated synaptically, by a bipolar electrode positioned in the white matter at the edge of layer 6, radially below the soma (outside the field of view), to produce an action potential. Twenty-one trials were averaged. Optical signals corresponding to evoked action potentials were clearly recorded from both soma (the largest signals from the soma are eliminated from the figure for clarity) and processes.

Comparison of a Nerve Gas Detoxifying Enzyme from Squid and from *Pseudomonas diminuta*

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and John E. Walker¹

Potent acetylcholinesterase (AChE) inhibitors such as DFP, $(\text{C}_2\text{H}_5\text{O})_2\text{P}(=\text{O})\text{F}$, and Soman, $\text{CH}_3(\text{C}_2\text{H}_5\text{O})\text{P}(=\text{O})\text{F}$, are hydrolyzed and detoxified by an enzyme in the nerve tissue of squid and other cephalopods (1). A second generation of these highly toxic compounds, typified by VX, $\text{CH}_3(\text{C}_2\text{H}_5\text{O})\text{P}(=\text{O})\text{SCH}_2\text{CH}_2\text{N}(\text{iC}_3\text{H}_7)_2$, and Tetriso, $(\text{iC}_3\text{H}_7\text{O})_2\text{P}(=\text{O})\text{SCH}_2\text{CH}_2\text{N}(\text{iC}_3\text{H}_7)_2$, are not hydrolyzed by the Soman-hydrolyzing enzyme from squid, termed "squid type" organophosphorus acid anhydrolase (OPAA). Recently we reported an organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* that hydrolyzes VX and Tetriso, and also DFP and Soman (2). Soman and VX are major components of now unwanted stockpiles of the so-called "nerve gases" (3).

Although squid type OPAA will not hydrolyze VX or Tetriso, these compounds may occupy the active site of the squid enzyme and thus inhibit its hydrolysis of DFP or Soman. Similarly, although the *Pseudomonas* OPH hydrolyzes all four compounds, VX or Tetriso might inhibit the hydrolysis of DFP or Soman, or DFP or Soman might inhibit the hydrolysis of VX or Tetriso. While the findings of such enzymes, seemingly without natural substrates, pose interesting fundamental questions that may be explored by combinations of these potential substrates and inhibitors, the disposal of mixed stockpiles of these nerve gases, as for example in the Gulf War aftermath, also poses practical questions.

Although Soman and VX are not readily available, findings with DFP and Tetriso are applicable to the nerve gas combinations, i.e., Soman and VX. To these ends, we have determined the enzymatic hydrolysis rates of DFP (and in a few experiments, Soman) in the presence and absence of Tetriso, and of Tetriso in the presence and absence of DFP (and in a few experiments, Soman). This is possible because the hydrolysis of DFP and Soman can be determined with a fluoride-sensitive electrode (1) even in the presence of Tetriso, and the hydrolysis of Tetriso can be determined with DTNB by the "direct" Ellman reaction (2, 4), even in the presence of DFP or Soman.

In a typical experiment of the first kind, a fluoride-sensitive electrode was placed in a stirred solution of 1.9 ml 0.025 M Hepes buffer (pH 7), 1.5 ml 0.01 M Tetriso, and 1.5 ml 0.01 M DFP. After determining the non-enzymatic rate, 0.1 ml OPH enzyme was added. The ensuing fluoride release was compared to an identical reaction in which Tetriso was replaced by buffer. In an identical experiment of the second kind, buffer, Tetriso, and DFP (1.06 ml of each), and 0.2 ml DTNB (1) were mixed in two cuvettes. Absorbance at 412 nm was noted (zero during 5 min), 0.1 ml OPH was added to the reaction vessel, and readings were continued. The absorbance was compared to an identical reaction in which DFP was replaced by buffer.

Table 1

Enzymatic hydrolysis of organophosphorus nerve gases singly and in pairs

Substrate (inhibitor)	Activity (% inhibition) of	
	OPH from <i>Pseudomonas diminuta</i>	OPAA from Squid (<i>Loligo pealii</i>) nerve
DFP	100	100
DFP (Tetriso)	87 (13%)	96 (4%)
Soman	31	26
Soman (Tetriso)	29 (6%)	26 (0%)
Tetriso	0.53	0
Tetriso (DFP)	0.02 (96%)	Not measurable
Tetriso (Soman)	0.36 (32%)	Not measurable

¹ Arbitrarily set at 100.

² No readable results, even with a 50-fold excess of enzyme.

The results of triplicate determinations (std. dev., <5%) are given in Table 1. Because the data are electrode and spectrophotometer readings, and because the two enzyme sources were purified to different degrees, always with respect to DFP as substrate, those results (line 1 of Table 1) were set to 100. All of the *Pseudomonas* readings were significantly greater than zero by the expedient of increasing the amount of enzyme, increasing the instrument sensitivity, or both; but with Tetriso in the role of substrate, a 50-fold increase of the amount of OPAA over that used with DFP, and full-scale absorbance set at 0 to 0.1, there was still no evidence of Tetriso hydrolysis by the squid enzyme.

The results in Table 1 were obtained with 3×10^{-3} M DFP, or Soman, or Tetriso, whether singly or in combination. They suggest that the affinity of the active site of OPH is greater for DFP, and probably Soman, than for Tetriso and, by analogy, VX. To explore this notion with the *Pseudomonas diminuta* OPH only, activities were determined over the concentration range of 5×10^{-3} to 10^{-4} M. For Tetriso as substrate, $K_M \approx 5 \times 10^{-3}$ M; with DFP as the potential inhibitor, $K_i \approx 2 \times 10^{-4}$ M. In the reverse experiment, with DFP as substrate, $K_M \approx 8 \times 10^{-5}$ M, and with Tetriso as inhibitor, $K_i \approx 3 \times 10^{-3}$ M. The inhibitions appear to be competitive but the concentration ranges, dictated by solubilities and the sensitivities of the methods, make both the constants and the nature of the inhibitions subject to some uncertainty. At a fundamental level, the active site of the *Pseudomonas* OPH appears to bind the P-F compounds about 50 times more strongly than the P-S-alkylamino group. The squid OPAA does not bind this latter group at all. At a practical level, these results suggest that additional hazards may be encountered in attempting to use enzymatic means for the disposal of mixed nerve gas stockpiles (3).

¹ U.S. Army Natick RD&E Center, Natick, Massachusetts 01760.

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Reference: *Biol. Bull.* 193: 263–264, (October, 1997)

Aspects of Lead Toxicity and Habituation in *Hermisenda* Learning

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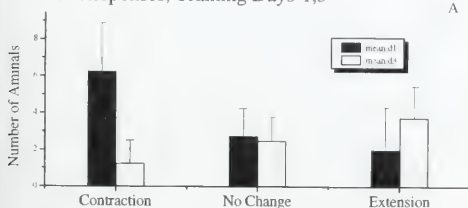
Lead toxicity remains a threat to human health and cognitive development (1). Investigating this multifaceted problem is difficult because lead lacks a single definable mechanism of action. The multiplicity of action sites confounds the straightforward

development of a single hypothesis to explain all the reported data (2, 3, 4).

Hermisenda has served as a neurobiological model for learning and memory studies for over 25 years. Many of the physiological properties underlying the behavioral modification this marine mollusc undergoes as a result of associative (Pavlovian) conditioning have been elucidated (5, 6). In an effort to demonstrate that this animal can be an effective model system for studying lead toxicity, we are testing various aspects of lead on the behavior and learning capabilities of *Hermisenda*. This report includes data on how lead exposure affects training acquisition and habituation by *Hermisenda*. It represents a portion of the data supporting the hypothesis that the behavioral alterations measured in lead-exposed animals reflect alterations in central nervous system processing and are unlikely to be caused by some general physiological impairment or loss of motor ability.

Animals (Sea Life Supply, Sand City, CA) were received

I. Mean Responses, Training Days-1,3



II. Training Acquisition

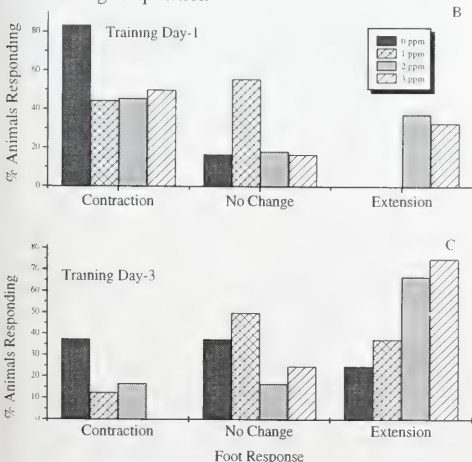


Figure 1. (A) I. Mean responses, training Days-1, 3: Graph depicts the mean responses of all animals to agitation on training Day-1 and Day 3. Irrespective of lead exposure (data pooled for lack of treatment effects), agitation elicited greater positive responses on Day-1; by Day-3, significant evidence of habituation was present. However, when these same animals were tested on Day-4 as per standard operating procedures, 78% of the control animals contracted with light alone, but only 33% of the 3 ppm PbAc-exposed animals contracted (normal data; see Kuzirian et al. (10)). (B) C. II. Training acquisition. Graphs illustrate the degree of response to 5 trials of paired light/agitation incurred by animals exposed to 0, 1, 2, and 3 ppm PbAc at the beginning of training Day-1, and Day-3. On training Day-1 (B), 83% of the 0 ppm PbAc controls contracted, whereas none of that group or the 1 ppm lead-exposed animals lengthened the foot in normal locomotion. Conversely, on training Day-3 (C), the percentage of control animals that exhibited a positive response dropped to 37.5% but the percentage of those animals extending the foot in response to agitation increased to 25%. Animals with higher lead exposures continued their trend of foot extension, raising the response rate to 66%–75%. The phenomenon demonstrated is habituation to the unconditioned stimulus (UCS), agitation. The habituation response was greater in the animals exposed to the higher concentrations of lead.

and adapted to laboratory conditions for 3 days. Control animals remained in natural seawater (NSW) while test animals were exposed to 1, 2, and 3 ppm (mg/l) of lead acetate (PbAc) ($n = 32$; $\times 8$ animals/condition). Routine training consisted of 30 presentations of paired light (condition stimulus; CS) and agitation (uncondition stimulus; UCS) for 3 days. On the 4th day, the animals were tested for their response to the CS alone (7). The first five to six training responses of the animals were videorecorded and measured at 1-min intervals. The initial and test responses were recorded as foot contraction (shortening of body length), no change in length, or foot (body) extension.

The mean responses of all animals to agitation (UCS) were compared between training Day-1 and Day-3. The results (Fig. 1A) demonstrated a decline in response to agitation irrespective of lead exposure. On Day-1, a significant number of animals contracted upon agitation, but by Day-3, the majority of the animals remained in the body extension phase of normal locomotion.

When the data were analyzed for lead effects (Fig. 1B, C), on Day-1 of paired training, 83% of the 0 ppm (NSW) animals contracted in response to the onset of agitation, and none of them (0%) extended the foot (body length) as in normal locomotion or motor functioning. Of the 1, 2, and 3 ppm PbAc-exposed animals, 44%–50% of them contracted, whereas 33%–37% of the remaining 2, 3 ppm lead-treated animals actually extended in body length, the normal response to light alone. By training Day-3, only 37.5% of the control animals contracted with agitation, but 66%–75% of the 2, 3 ppm PbAc-exposed animals extended their foot length; an approximate doubling of the Day-1 extension rate. The lead-exposed animals were moving normally in their fully extended body position, essentially unresponsive to the agitation stimulus. When the animals were tested on the 4th day with light alone (CS), 78% of the control animals contracted, whereas the lead-exposed animals had an average contraction rate of 33%, or $\approx 67\%$ non-responders (normal data, not graphed).

The decrease in percent contraction between the 1st and 3rd days of training shows habituation to the repeated presentation of the agitation stimulus over time (8). Actually, the 2, 3 ppm

lead-exposed animals demonstrated an even greater habituation, doubling the foot extension rate by training Day-3. These results mimic the foot extensions in response to light reported by Matzel *et al.* (9) for animals receiving unpaired light and agitation or light only. Of significance was the testing responses (degree of learning) demonstrated by both the controls and the lead-exposed animals. The results mirror precisely the behavioral data reported for lead at levels approaching 5 ppm (10). Lead concentrations as low as 1 ppm reduced the ability of *Hermisenda* to undergo Pavlovian conditioning. Thus, the increase in foot extensions, the locomotory patterns exhibited, and the lack of training effects further support the hypothesis that lead-exposed animals are not broadly physiologically impaired (depressed muscle and locomotory function, or metabolic output), but rather their behavior demonstrates an impairment in central nervous system function related to sensory input, signal processing, or both.

Proper vestibular synaptic input onto the medial B-photoreceptor is necessary for the acquisition and modulation of learning in *Hermisenda*. Decreased vestibular output could be responsible for the lack of foot contraction and the increased foot length of normal locomotion (11). Detailed electrophysiological studies are planned to elucidate possible lead effects on the photic-vestibular pathways.

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Righting Response and Escape Response in *Opsanus tau* Are Temperature Dependent

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We sought to establish baseline values for two basic reflex behaviors and for ventilation rate in the toadfish, *Opsanus tau*, over a range of temperatures. Righting response and escape response were chosen as measurable reflexes indicative of activity level in these animals.

Between September, 1996, and January, 1997, ten fish were held in an isolated tank at ambient temperature in the Marine Resources Center at the Marine Biological Laboratory in Woods Hole, Massachusetts. The animals were subjected to a series of behavioral tests as the ambient water decreased in temperature. Fish were acclimated a minimum of 24 hours at each temperature before testing.

Tests were conducted in an experimental tank, 65 cm wide $\times$ 100 cm long $\times$ 10 cm deep, and filled with water at the same temperature to which the animals had been acclimated. Trials were recorded with a video camera mounted on a tripod above the tank. During testing, a single animal was placed in the tank, and righting response, escape response, and the ventilation rate were quantified consecutively.

The righting response was measured as follows. A fish was held ventral side up for several seconds and then released. The animal could then attempt to right itself. These trials were scored on an incremental scale of five levels based on the number of attempts required by the animal to right itself and on the effort exerted (4, successful on first attempt with accessory tail movement; 3, successful on first attempt with minimal tail movement; 2, successful after multiple attempts; 1, unsuccessful attempts; and 0, no attempts).

The escape response was quantified by probing and pinching the animal posterior to the last dorsal spine with toothed forceps. Three levels of pressure (probe, hold, hold, and pull) were applied to all animals with a 30-s recovery period between stimuli. From these three trials, a single escape response score was generated. Escape responses were divided into high velocity (≥ 20 cm/s) and low velocity (< 20 cm/s) responses. The stimulus of least force to which the animal responded at high velocity was the basis for scoring. If the animal did not exhibit a high velocity response to the first two levels of stimulus, the score was determined using the strongest stimulus applied (animal held and pulled with forceps). In general, scoring, using an incremental scale, was based on response velocity relative to the type of stimulus eliciting that response (4, escape velocity ≥ 20 cm/s when touched with forceps; 3, same velocity when held with forceps; 2, same velocity when held and tugged with

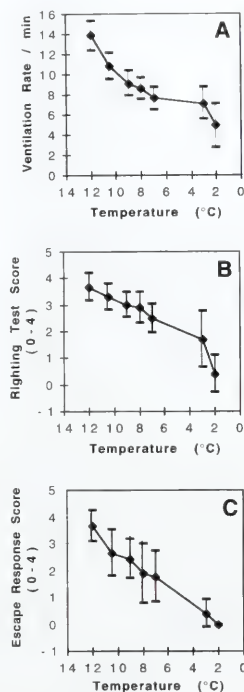


Figure 1. A. Effect of temperature on ventilation rates of *Opsanus tau* measured in operculum beats per minute (mean $\pm$ S.D.). B. Effect of temperature on righting response levels as measured by the ranking system described in text (mean $\pm$ S.D.). C. Effect of temperature on escape response levels as measured by the ranking system described in text (mean $\pm$ S.D.).

forceps; 1, velocity < 20 cm/s when held and tugged; and 0, no escape response).

At the conclusion of the test, following a one-minute recovery period, we determined the ventilation rate by counting opercular movements for one minute. Afterwards, the fish were placed in a holding barrel until tests had been completed for all animals. These procedures were repeated for the same ten animals at seven temperatures between 12 and 2°C.

Activity levels for all tests show decreases with temperature. Also, an activity threshold in the toadfish appears at 3°C. At this temperature, activity levels begin dropping rapidly to an

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activity level of nearly zero, reached at 2°C. This decrease is at a faster rate than in any other part of the temperature range for both righting response and ventilation rate. This activity threshold is consistent with the torpid state of animals noted at the lowest temperatures in captivity and with burrowing behavior that takes place in the wild between 10 and 5°C in preparation for cold weather.

These data, which establish normal values for activity level over a wide range of temperature, in conjunction with additional data at higher temperatures (unpub. data) will be beneficial in choosing healthy animals for research. Medically important research on toadfish has been conducted on the vestibular system of the inner ear (1, 2, 3) and on the pancreas (4). Recent studies, however, have been compromised by unhealthy fish. These behavioral experiments should help to establish a series

of baseline behavioral measurements that will allow researchers to select the most robust fish for physiological studies.

We thank Stephen Highstein of the Washington University School of Medicine and Edward Enos of the Marine Resources Center for their support of this project.

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Physiological Stress Elevates Hemolymph Levels of Methyl Farnesoate in the Green Crab *Carcinus maenas*

Donald L. Lovett (The College of New Jersey, Ewing, New Jersey 08628), Patrick D. Clifford¹, and David W. Borst²

Methyl farnesoate (MF), a sesquiterpene from crustaceans, is structurally similar to insect juvenile hormone. Thus it may have a role in regulating crustacean metamorphosis, reproduction, and behavior (1, 2). In preliminary studies, we measured the levels of MF in the hemolymph of several crustaceans daily and at different times during the molting and reproductive cycles. We found that MF levels often were elevated in animals that had been stressed by handling or disease. In this study, we have applied several types of physiological stress to green crabs and have examined the effects of these stressors on MF levels.

Green crabs, *Carcinus maenas*, were collected from wild stocks around Woods Hole, Massachusetts, and were maintained in running seawater (approximately 32 ppt salinity, 22°C, and saturated with O₂). The effects of changes in salinity, temperature, and oxygen concentration were tested in these experiments, and the severity of these stressful conditions was limited to that typically encountered by the organism in its environment (3). None of the conditions had any long-lasting adverse effect on the animals (some animals were monitored several days after their return to running seawater; MF levels had returned to control levels). Only intermolt males were used. To test the effects of salinity and temperature, animals were acclimated for at least 10 days [acclimation times reviewed in (4)]; both control and treatment animals then were transferred ($t = 0$) directly into recirculating, temperature-regulated tanks with a biological

filter and containing water at the appropriate salinity and temperature. Between 25 and 100% of the tank was exchanged daily, depending upon the number of crabs in a tank. Both control and treatment animals were removed and analyzed after the same exposure times. For hypoosmotic stress, animals were exposed to seawater diluted to 5 ppt with deionized water. For temperature stress, the water in the tanks was warmed to 32°C or chilled to 14°C. To test the effects of anoxia, animals were placed in a covered 1 or 2 l flask containing seawater saturated previously for 30 min with nitrogen gas; saturation was maintained by bubbling with nitrogen throughout the experiment. Control flasks were bubbled with air. Oxygen concentration in treatment flasks was approximately 0.25 ppm, while that in control flasks was approximately 7.0 ppm. Hemolymph levels of MF were determined by HPLC as previously described (5).

Three physiological stresses (increased temperature, anoxia, and decreased salinity) were followed by a significant ($p < 0.05$) increase in the level of hemolymph MF (Table I). In contrast, cooling had no effect on MF levels, and transfer of animals acclimated to dilute seawater (10 ppt salinity) to full-strength seawater was followed by a drop in MF to basal levels.

Significant increases in MF levels were observed in both control and treatment animals during the first hour of the experiment (data not shown); this might be due to stress associated with handling. In any event, MF levels in controls typically returned to background levels by 2 h after exposure. MF levels in treatment animals exposed to increased temperature or anoxia increased significantly over levels in control animals in less than 2 h, but data were not reported because of the effect on controls during this time. While anoxia and increased temperature elicited increases in MF levels within 2 h, a significant

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Table I

Effects of physiological stress on the concentration of methyl farnesoate in the hemolymph of green crabs *Carcinus maenas*

Type of stress	n (control, treatment)	Time ^a (h)	Concentration of methyl farnesoate (ng/ml hemolymph) (mean $\pm$ SE)	
			Control	Treatment
<i>Thermal stress</i>				
14 to 32°C	6, 6	2	4.2 $\pm$ 0.7	13.7 $\pm$ 1.4**
23 to 14°C	6, 5	2	4.2 $\pm$ 2.5	1.8 $\pm$ 0.7
<i>Anoxia</i>				
7 to 0.25 ppm O ₂	6, 6	2	0.4 $\pm$ 0.4	7.4 $\pm$ 0.7**
<i>Osmotic stress</i>				
32 to 5 ppt salinity	6, 6	8	3.9 $\pm$ 1.8	47.3 $\pm$ 13.7**
10 to 32 ppt salinity	6, 6	6 ^b	32.2 $\pm$ 11.9	1.5 $\pm$ 0.9**

** = means of treatments are significantly different from the means of controls ($p < 0.05$; t -test).

^a Time from the onset of stress to the measurement of significant change in MF levels.

^b Animals not examined prior to 6 h.

response to hypoosmotic stress appeared only after 8 h. A delayed response to decreased salinity may be associated with a delayed change in hemolymph osmolality, as has been reported for portunid crabs (6, 7). Levels of MF in animals exposed to elevated temperature declined to control levels within a few days, suggesting that acclimation had occurred. In contrast, levels of MF in animals transferred to low salinity remained elevated after 2 weeks. Levels of MF in animals exposed to anoxia remained elevated during the time periods examined (up to 4 h).

In preliminary experiments, we also tested the effect of similar stresses on MF levels in *Libinia emarginata* and *Callinectes*

sapidus. In these species, levels of MF also increased following transfer to increased temperature or decreased salinity (data not shown).

We have shown that the hemolymph level of MF can increase rapidly and markedly in response to a variety of stresses. Thus, experiments testing the function of this compound must be designed with care. Increase in MF levels may be a direct effect of the stress, or instead may be part of an adaptive mechanism to the stress. Given that levels of MF eventually return to basal levels in response to some stresses (*i.e.*, following handling of the animals or during acclimation to elevated temperature) and yet remain elevated in animals acclimated to low salinity, at least two types of responses or mechanisms may involve MF. Further study of possible involvement of MF in responses to stress, particularly to salinity change, will be the subject of future investigations.

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Relationship of Methyl Transferase Activity and Methyl Farnesoate Levels in the Spider Crab, *Libinia emarginata*

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Methyl farnesoate (MF) is a sesquiterpene produced by the crustacean mandibular organ (MO). Although MF is structurally similar to insect juvenile hormone, the functions of this compound in crustaceans are still not completely resolved (1). One

approach to studying the functions of MF has been to measure the *in vitro* synthesis of MF by MOs from animals in different physiological states (2). Another approach has been to measure the levels of MF in the hemolymph (3, 4). Finally, we have recently described an assay for farnesoic acid *O*-methyltransferase (methyl transferase; MeT), the enzyme in the MO that catalyzes the final step of MF production (5). In this study, we compare two methods of estimating MO function (MF synthesis and MeT activity) with the hemolymph level of MF in the spider crab, *Libinia emarginata*.

Spider crabs were obtained from the Marine Resources Cen-

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ter at the Marine Biological Laboratory. The exoskeletons of these animals were completely abraded or nearly so, a feature that has been correlated with higher hemolymph levels of MF in this species (4). The crabs were maintained in seawater and fed squid *ad libitum* until used. Some animals were stressed by immersion in hypoosmotic (20 ppt) seawater for 12 h, a procedure that elevates hemolymph levels of MF in the green crab, *Carcinus maenas* (6).

MF levels in the hemolymph were measured as previously described (3) and are expressed as pmoles/ml of hemolymph. MF synthesis was measured as previously described (2) using a modified culture medium (Dulbecco's modified Eagle medium made up at a 1.5-fold concentration and supplemented with 29 mM glucose, 4 mM NaHCO₃, 395 mM sucrose and 25 mM HEPES, pH 7.4) containing [³H]-methionine (NEN, 200 mCi/mmol, 20 μ Ci/ml, final specific activity = 38 mCi/mmol). The synthetic activity of each MO was calculated as pmoles MF produced during the 2-h incubation. MeT activity was measured using the assay conditions previously developed for the lobster, *Homarus americanus* (5). MeT activity measured in MOs from *L. emarginata* increased linearly with time (up to 40 min) and increasing enzyme concentration and had an apparent Km of about 1 μ M. An incubation period of 30 min was used in the standard MeT assay, and activity was calculated as pmoles MF produced per MO or per pair of MOs.

Individual MOs from animals [$n = 11$; carapace length (CL) = 75.9 ± 1.6 mm] maintained in full strength seawater synthesized between 21 and 1071 pmoles MF during the 2-h incubation, with an average synthesis of 550 ± 56 ($n = 22$) pmoles MF. The amounts of MF synthesized by MOs from the same animal were poorly correlated ($r^2 = 0.015$), with the amount synthesized by the less active gland being 5 to 98% of that observed in the more active gland. This observation confirms an earlier report that MOs are asymmetric in their synthetic activity (7). The mean concentration of MF in these animals was 71.6 ± 14 pmoles/ml of hemolymph. The correlation between hemolymph levels of MF and either total MF synthesis (the sum of the synthesis observed in both MOs from a single animal) or MF synthesis by the more active gland was low ($r^2 = 0.284$ and 0.316 , respectively).

The MeT activity of individual MOs from a second set of crabs ($n = 11$, CL = 61.6 ± 1.4 mm) maintained in full-strength seawater varied from 1 to 99 pmoles/MO; the mean MeT activity was 16.2 pmoles/MO (± 5.0 ; $n = 22$). Although the MeT activity of the two MOs from some crabs varied (the less active MO had from 12 to 99% of the MeT activity observed in the more active MO), there was a good correlation in the activity

measured in the MOs from the same animal ($r^2 = 0.689$). The average hemolymph level of MF in these animals was 144 ± 39 pmoles/ml of hemolymph. MeT activity was strongly correlated with hemolymph levels of MF ($r^2 = 0.835$).

MeT activity and hemolymph levels of MF were also measured in crabs that had been osmotically stressed for 12 h ($n = 8$; CL = 63.1 ± 1.0 mm). Hemolymph levels of MF were significantly higher ($p < 0.05$; t -test) in osmotically stressed animals (1272 ± 448 pmoles/ml; $n = 4$) than in control animals (240 ± 104 pmoles/ml; $n = 4$). The level of MeT activity in osmotically stressed animals (238 ± 95 pmoles/MO, $n = 3$) was also significantly higher ($p < 0.02$; t -test) than in controls (23.1 ± 7.4 pmoles/MO, $n = 3$). In addition, MeT activity was strongly correlated with hemolymph levels of MF ($r^2 = 0.941$).

In conclusion, MeT activity shows a strong correlation with hemolymph levels of MF whereas the synthesis of MF by MOs incubated *in vitro* does not. This may reflect a change in the rate of MF synthesis during the *in vitro* incubation due to the absence of compounds that regulate MO function *in vivo*. Thus, MeT activity may be a more useful marker of MO activity in crustaceans. Finally, the strong correlation of MeT activity with hemolymph levels of MF in both animals maintained in full-strength and hypoosmotic seawater suggests that this enzyme may play an important role in the regulation of MF synthesis in the MO.

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Identification of Toadfish-Pathogenic Bacteria Based on a Comparative Molecular Approach

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Opsanus tau (oyster toadfish) is an important laboratory animal at the Marine Biological Laboratory (MBL); they are used as a model for studying the mechanisms of hearing and balance.

A new disease, bacterial pericarditis, which is caused by a gram negative bacterium, has caused mortalities in the Marine Resources Center (MRC). Determining the identity of this pathogen, which has not been previously seen in toadfish (1), is important to the health management program for this species. A rapid and accurate method, based on the 16S rRNA gene, for the identification of bacterial pathogens was used to address this problem (2, 3).

Bacterial strains ($n = 15$) were isolated from individual infected toadfishes ($n = 12$) during the outbreak of bacterial pericarditis that occurred from July 1996 to April 1997 at the MRC. Brain heart infusion agar (BHIA; Difco, Detroit, MI) with 1.2% NaCl, was used to isolate and purify bacterial cultures. All isolates were first tested for antibiotic resistance. BHIA antibiotic plates, including ampicillin (50 $\mu\text{g/ml}$), kanamycin (25 $\mu\text{g/ml}$), tetracycline (10 $\mu\text{g/ml}$), and vancomycin (100 $\mu\text{g/ml}$), were used. The plates were incubated for 24 h at room temperature.

Bacterial DNA was isolated from each isolate using the bead beater procedure and standard phenol extraction. Universal prokaryote primers specific to the ends of prokaryotic 16S rRNA were used to amplify this gene. DNA hybridization (checker board) was applied to the 16S rRNA of all isolates using 16S rRNA complementary probes: universal prokaryotic, enteric, betas, deltas, sulfate reducing bacteria, flavobacteria, gram positive low GC, and spirochete.

To screen the PCR products and compare the 16S rDNA in all isolates, restriction fragment length polymorphism analysis (RFLP) was performed using four tetrameric restriction enzymes, *HaeIII*, *HinPI*, *MspI*, and *RsaI* (4). To confirm the results obtained from the RFLP analysis, denaturing gradient gel electrophoresis (DGGE) was applied. DGGE primers were used to amplify the 16S rRNA gene, and the PCR products were run in a 40%–60% urea formamide gradient acrylamide gel. After the RFLP and DGGE comparisons, representative samples with a common RFLP DNA pattern and DGGE band positions were chosen for sequencing with an ABI automatic apparatus. Sequences obtained were compared to the non-redundant nucleotide database at the National Center for Biotechnology Information using the BLAST (Basic Local Alignment Tool) algorithm. Phylogenetic analyses were done according to Paster (5).

All isolates (#1–#15) tested for antibiotic resistance were susceptible to ampicillin, kanamycin, and tetracycline, and were resistant to vancomycin. If antibiotic treatment is needed, ampi-

cillin and tetracycline can be used on the infected fish. Kanamycin, however, is not recommended because spontaneous resistance develops. The resistance of the bacteria to vancomycin is not surprising because this antibiotic cannot penetrate the gram negative outer membrane (6).

All of the fifteen 16S rRNA genes from the bacterial isolates gave positive signals with enteric and universal probes, but no signal was detected with betas, deltas, SRBs, flavobacteria, gram positive low GC, or spirochete probes. This result indicates that all of the isolates tested by checkerboard hybridization can be classified as enteric bacteria. The comparative RFLP analysis of the 16S rRNA gene confirmed that all 15 isolates can be classified into two distinctive patterns: Group I includes isolates #1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14; and group II includes isolates #3, 7, 15. *HinPI* enzyme cut these 16S rRNA fragments more frequently than any other tested enzyme. DGGE analysis revealed that the group I isolates are identical. Sequence analysis of 16S rRNA genes of the group I isolates (#2, 4, 6, 14) indicates that they can be classified into the gamma purple cluster and are phylogenetically closely related to *Escherichia coli*. Current analyses suggest that the isolates could be a new species not yet described. Sequence analysis of 16S rRNA group II isolates (#3 and #15) indicates that they are phylogenetically close to the *Photobacterium* cluster. Photobacteria, commonly found in healthy marine fish (7, 8), are presumed to be contaminants.

The molecular analysis of group I bacteria (12 of 15) confirms that the predominant isolates are identical, and suggests that this coliform-like bacteria is the causative agent of the disease. Work is being conducted to fulfill Koch's postulates, and pulsed field gel electrophoretic (PFGE) analysis should provide additional information. Moreover, from the sequence data obtained, we will design a 16S rDNA primer that is specific for the etiological agent and can be used for the rapid detection of this pathogen.

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Bacterial Pericarditis: A New Disease of Toadfish

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The toadfish, *Opsanus tau*, is an important research animal used in the study of diabetes, muscle physiology, sound reception, and equilibrium. Toadfish are sediment dwellers that live inshore on rocky bottom and reefs. During the winter they migrate to deeper waters, bury themselves, and remain torpid till the spring (1). In the spring and summer, personnel of the Marine Biological Laboratory's Marine Resource Center (MRC) collect toadfish from various areas along the Massachusetts coast. These animals are kept over the winter to supply specimens for research.

Toadfish captured during the spring and early summer of 1996 were held in ambient water until September, then were placed in treated water held at about 10°-15°C through the winter. Beginning at the end of July 1996 and continuing until April 1997, mortalities due to a new disease, termed here bacterial pericarditis, were identified in the adult populations of toadfish held at the MRC. Over the 8-month period, an epidemic level of this disease, 51% (24/47), was identified in the dead or moribund toadfish submitted for necropsy. At necropsy, 33% (8/24) of the fish with bacterial pericarditis exhibited swollen "coconut belly" abdomens that contained up to 140 ml of serosanguinous fluid that clotted when exposed to air (Fig. 1A). Internally, 79% (19/24) of the affected fish showed an accumulation of up to 5 ml of white/tan to pink, thick, flocculent pericardial exudate (Fig. 1B). Occasionally, the exudate appeared more diptheritic and encased the heart. Multifocal discrete white/tan nodules were common in the myocardium of the ventricle. Eighty-three percent (20/24) of the fish showed nodules in the liver that ranged from 6 cm to <1 mm (Fig. 1C). In 4% (1/24) of the cases, a large nodule at the hilus of the liver, without apparent pericardial component, was associated with ascites. Nodules (2 cm to <1 mm diameter) were also identified in the kidney (17%, 4/24), in both male and female gonads (42%, 10/24) and in the male accessory sex glands (17%, 3/18) of several fish. In 8% (2/24) of the cases, the air bladder was filled with thick, flocculent, white/tan exudate.

Tissues were removed at necropsy, processed in paraffin, and stained with hematoxylin and eosin using standard methods (2). Histologically, the pericardial inflammation consisted of

necrotic cells, macrophages and lesser numbers of heterophils and lymphocytes. Rod-shaped bacteria were abundant both within inflammatory cells and free in the debris (Fig. 1D, 1E). Multifocally fibroblastic proliferation forming granulation tissue was noted at the junction of the pericardial exudate and the epicardium. Multifocal discrete nodules seen in many organs were characterized by central liquefactive necrosis intermixed with variable numbers of bacteria. Many macrophages and a lesser number of heterophils surrounded and isolated the necrotic debris from the surrounding organ parenchyma, but only rarely were encapsulating fibroblastic-like macrophages seen (3) (Fig. 1F). In addition, the livers showed severe edema accompanied by dilation of the space of Disse, bile stasis within canaliculi and hepatocytes, and atrophy of the hepatic cords.

The most characteristic gross lesions identified in this disease were pericarditis and hepatic nodules. Pericarditis and myocarditis caused poor myocardial function resulting in secondary congestion of the liver. In animals showing a more chronic and severe pericarditis, ascites resulted in the gross signs of "coconut belly." Rarely, large nodules at the hepatic hilus resulted in hepatic blood stasis and ascites. Bacterial pericarditis devastated the adult toadfish population held at the MRC over the winter of 1996-1997. This disease, characterized grossly by severe pericarditis, has not been previously reported in toadfish or other fish and has not been previously identified in MRC toadfish. The origin of the bacterium causing this disease is not known but has been classified as *E. coli*-like. Work is being conducted to further classify the bacterium.

We thank the Elizabeth Wadman and the staff of the MRC for their support and help.

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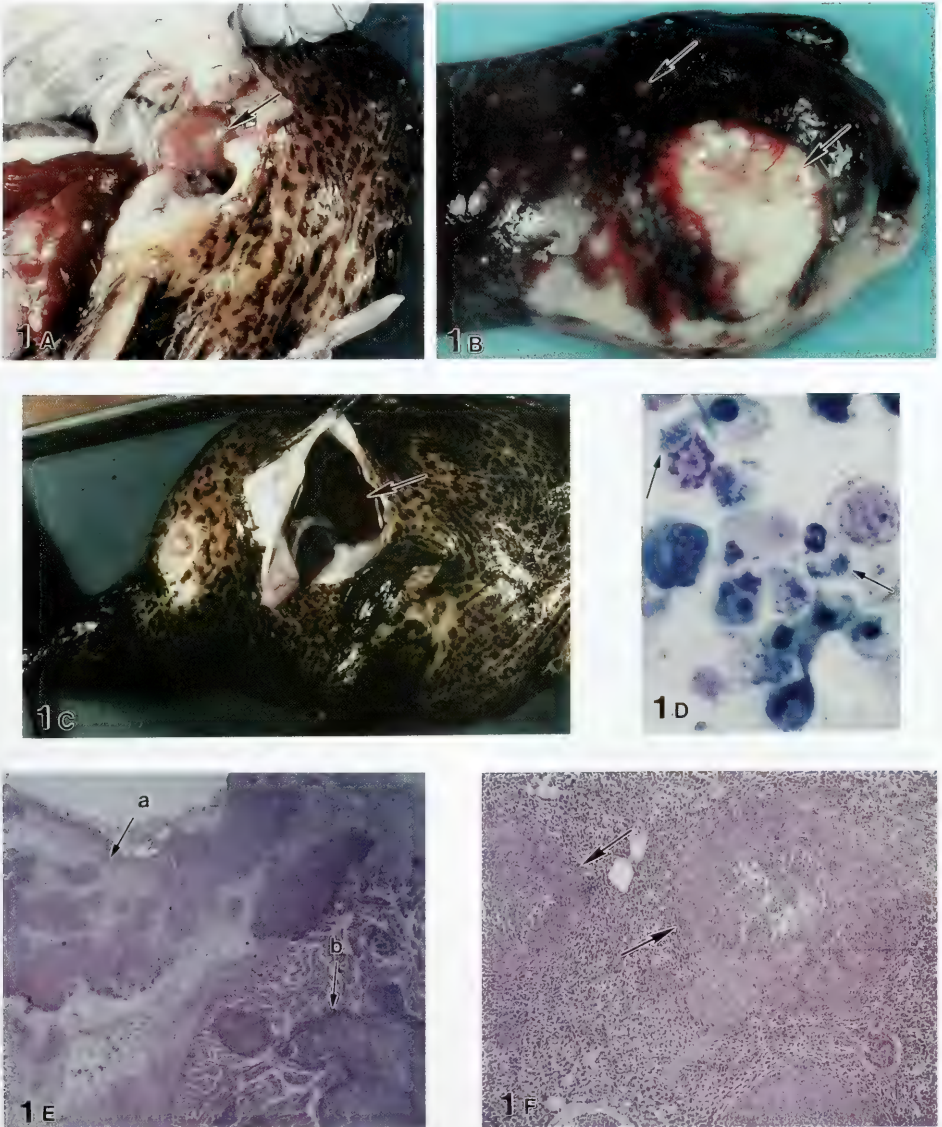


Figure 1. (A) The pericardial cavity of the toadfish is opened and contains the unopened pericardial sac filled with exudate. A focal myocardial nodule (arrow) can be identified. (B) Nodules are present in the liver parenchyma (arrows). (C) The abdominal wall has been incised and the serosanguinous ascites is seen (arrow). (D) The Giemsa-stained smear of pericardial exudate shows abundant bacteria in inflammatory cells (arrows) (250 $\times$). (E) Pericardial exudate (a) and myocardial nodules (b) are identified in the ventricle of the heart the (paraffin section stained with hematoxylin and eosin, 10 $\times$). (F) Subacute multifocal liquefactive granulomata are present in the liver parenchyma (arrow) (paraffin section stained with hematoxylin and eosin, 10 $\times$).

Differential Leucocyte Counts in the Toadfish, *Opsanus tau*: Insignificant Variation with Seasonal Temperature

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The toadfish, *Opsanus tau*, is a temperate marine fish that serves as a valuable model for biomedical experimentation. At the Marine Biological Laboratory (MBL), wild-caught toadfish in various states of health are placed in the captive environment for use by researchers. A standard set of health measures is essential, therefore, if only healthy individuals are to be selected for experimentation. One valuable measure is the differential leucocyte count, which indicates the percentage of white blood cell types in circulation. Stressful conditions such as temperature change and infectious disease are known to have profound effects on leucocyte counts in fish when compared to established normal percentages (1). The normal percentages of leucocyte subtypes have never been published for the toadfish. We hypothesized that leucocyte values for this species would change as a function of temperature. Determination of both leucocyte subtypes and the seasonally expected changes in blood parameters would, in turn, allow us to interpret the meaning of such changes more accurately in both healthy and diseased toadfish.

Forty-eight adult toadfish averaging 742 gm in weight and 32.7 cm in length were captured from local waters, tagged with a plastic T-Tag (Floy Tag Co., Seattle, WA) and held in the Marine Resources Center (MRC) in tanks supplied with ambient seawater. After a 2-week acclimatization period, biweekly blood samples (0.3 ml) were taken from the caudal vein with a 3/4", 23-gauge needle at six temperatures (15°, 12.5°, 11°, 8°, 7.1°, and 5.5°C) during 12 weeks (Oct.–Dec. 1996) of declining ambient water temperatures. Fish were bled in the late afternoon (1600–1800 h) following light sedation by immersion in a 2% solution of MS-222 (Argent Labs, Redmond, WA) in seawater. Animals were fed daily *ad libitum* with squid or chopped clams. Hematocrit values were determined by the microhematocrit tube method. Blood films were prepared at the time of sampling and were stained with Wrights giemsa. Differential leucocyte counts ($n = 100$) were made by direct observation of duplicate smears by standard techniques (2).

Our results showed no temperature-related changes in leucocyte percentages. No significant changes with temperature were observed in packed red cell volume (hematocrit), which averaged 18%. The bleeding schedule was thus appropriate, because no signs of hypovolemic anemia were present. During the course of the study, tags became dislodged and the identity of individual fish lost. Results were, therefore, pooled and cell averages determined. The changes in averages relative to temperature were random, except in the case of monocytes (Table I). The standard deviations were very large and overlapped, suggesting the differences were not statistically significant. Monocytes ranging from

Table I

Differential leucocyte counts (mean $\pm$ 1 SD; $n = 100$) for the toadfish as a function of temperature

Temperature (°C)	Lymphocytes			Heterophils	Monocytes
	Small	Medium	Large		
15	14.7 $\pm$ 11.4	15.3 $\pm$ 10.4	12.1 $\pm$ 7.3	55.0 $\pm$ 16.2	2.1 $\pm$ 1.9
12.5	16.8 $\pm$ 1.2	24.0 $\pm$ 9.9	8.8 $\pm$ 5.1	50.0 $\pm$ 10.6	1.0 $\pm$ 0.5
11	26.8 $\pm$ 12.5	28.7 $\pm$ 11.7	8.5 $\pm$ 7.0	32.4 $\pm$ 15.0	2.9 $\pm$ 1.6
8	40.9 $\pm$ 11.9	23.5 $\pm$ 7.3	3.5 $\pm$ 3.4	29.4 $\pm$ 8.24	3.5 $\pm$ 2.6
7.1	11.5 $\pm$ 7.8	15.5 $\pm$ 7.0	3.9 $\pm$ 3.0	67.4 $\pm$ 11.9	1.9 $\pm$ 1.0
5.5	17.0 $\pm$ 8.8	28.5 $\pm$ 9.9	5.4 $\pm$ 2.5	46.2 $\pm$ 14.4	3.0 $\pm$ 1.8

only 0%–4%, had very narrow deviations, yet revealed temperature's lack of effect. Count averages give normal values of 19 ($\pm$ 8.9) % for small lymphocytes, 23 ($\pm$ 9.3) % for medium lymphocytes, 7 ($\pm$ 4.7) % for large lymphocytes, 48 ($\pm$ 12.7) % for heterophils, and 2 ($\pm$ 1.6) % for monocytes. These results represent the first published leucocyte counts for this species.

There are interesting comparisons to be made between the values determined for this fish and those published for other estuarine species. Low monocyte counts (<4%) are typical for most species (3). Interestingly, there was a notable absence of heterophil subtypes in the toadfish. Despite several modifications of the staining procedures, eosinophils and basophils were never discernible, which may be characteristic of this species. The blood of *Fundulus heteroclitus*, a fish encountered in the same habitats as the toadfish, shows zero counts for heterophils and monocytes while eosinophils and lymphocyte averages remain high (3). A new tagging technique (unpubl. data) will allow us to permanently identify individual animals in future studies, which will address clinically significant changes in leucocyte percentages in response to stress, disease such as bacterial pericarditis (4), and holding conditions.

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A Hemolytic Activity Secreted by the Endotoxin-Challenged Horseshoe Crab: A Novel Immune System Operating at the Surface of the Carapace

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The American horseshoe crab, *Limulus polyphemus*, releases 1–5 ml of a proteinaceous secretion at the surface of the carapace when challenged by intracardiac injections of bacterial endotoxin (lipopolysaccharide, LPS) administered at 0.1 µg/ml of blood. Female animals were used because LPS injection stimulates males to shed semen, which then contaminates the exudate. Blood volume is estimated as 1/3 of the total body weight of the animal. The secretion (exudate) is presumably the product of the hypodermal glands, whose ducts extend from the dermis to the surface of the carapace (1). The cytolytic activity of the secretion was assayed by the lysis of sheep erythrocytes using methods described previously (2). This hemolytic activity was evident at a 1:400 dilution of the crude exudate and was unaffected by chelation of divalent cations with ethylenediaminetetraacetic acid (EDTA) and inhibition of endogenous protease activity with α_2 -macroglobulin and phenylmethylsulfonylfluoride. Hemolysis appears to be mediated by a protein, because the hemolytic factor is non-dialyzable with a 10-kDa exclusion limit dialysis membrane, is heat labile, is sensitive to treatment at low (pH 2) and high (pH 12) pH, and is partially sensitive to trypsin. Seawater that ran off the surfaces of the unchallenged animal was not hemolytic. Animals that were injected only with the diluent for LPS (pyrogen-free 3% NaCl) failed to secrete the hemolytic exudate. SDS-polyacrylamide gel electrophoresis (reducing conditions) showed the LPS-elicited exudate to contain major protein bands at 126, 93, 83, 79, 48, 47, 26, 23, 17, 16, 15, and 6 kDa and a number of minor bands between 45 and 30 kDa. The pattern of proteins seen in SDS-PAGE shows that the exudate contained little or none of the abundant proteins of the plasma (e.g., α_2 -macroglobulin, hemocyanin, and C-reactive protein) and also contains no detectable quantities of the suite of proteins secreted by blood cells stimulated to undergo exocytosis by challenge with LPS or with the Ca^{2+} -ionophore, A23187 (3). The hemolytic activity partitioned by gel exclusion chromatography on Sephadex G-100 resin with an apparent molecular mass of 25–30 kDa. The most active fractions from the G-100 column were enriched in a major protein band seen in SDS-PAGE (reducing conditions) at 47 kDa, a suite of minor bands between 45 and 30 kDa, and a protein band at 23 kDa. The protein responsible for hemolysis failed to bind to Mono Q anion-exchange resin at low salt concentrations (approx. 50 mM NaCl), consistent with the possibility that it is a basic protein. SDS-PAGE (reducing conditions) showed that the active fractions from the Mono Q column contained five prominent proteins as a band at 45 kDa, a triplet of bands at 31 kDa, and a band at 23 kDa.

The plasma of *Limulus* contains a potent Ca^{2+} -dependent hemolytic activity that is mediated by the protein limulin, a 300-kDa sialic acid-binding lectin that is a member of the pentraxin family of proteins (4). The hemolytic activity of the secretions of the hypodermal glands cannot be mediated by limulin: i.e., the activity is Ca^{2+} -independent, is not removed by exposure to fetuin-Sepharose (which removes limulin and abolishes the hemolytic activity of *Limulus* plasma (4)), and it partitions by molecular-exclusion column chromatography at a molecular mass about 1/10 of that of limulin. Crude exudate does not appear to contain hemagglutinins, because the sheep erythrocytes in the hemolytic test system do not cluster as visible aggregates. The blood cells release membrane active antimicrobial factors, the tachyplesins, big defensin, and anti-lipopolysaccharide factor, which are, respectively, a family of 17-residue peptides (5) and proteins of 8.6 (6) and 11.6 (7) kDa and are, thus, too small to be responsible for the hemolytic action of the exudate.

The surfaces of most solid objects exposed to ocean waters soon become encrusted with a diverse array of sessile animals, plants, and microbes. In contrast to the solid inanimate objects with which they share the environment, most macroscopic marine organisms, even long-lived ones, are typically only sparingly decorated with fouling organisms. We suggest that the endotoxin-elicited secretion of *Limulus* functions as an anti-biological agent that discourages colonization of the carapace of this species by sessile prokaryotic and eukaryotic organisms.

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Biosynthetic Induction of a Secondary Metabolite by a Marine Bacterium under Nutritional Stress: Potential Role of the Incomplete Oxidation of an Organic Acid

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Natural products have historically been an important source of new pharmaceutical compounds. Traditionally, the major source of these compounds have been secondary metabolites of terrestrial plants and microorganisms. More recent efforts by our laboratory and others have focused on the virtually untapped potential of the marine environment. We have concentrated on isolating marine bacteria from a broad variety of marine ecological niches for the purpose isolating new natural products. To fully exploit this resource, we have manipulated nutrient conditions in an attempt to induce secondary metabolite production in strains that normally produce little. The present report describes such an induction caused by the addition of the five carbon organic acid, α -ketoglutarate, to minimal nutrient marine-based medium. We also describe a simple method for testing crude extracts for antimicrobial activity combined with thin layer chromatography (TLC) characterization of the extracts.

In our experimental model for carbon utilization in marine microorganisms, nutrient-limiting environmental conditions will not allow cellular metabolism to shuttle excess carbon into biosyntheses for growth, but will instead shuttle it into the production of secondary metabolites. This secondary metabolic production would likely be tightly coupled to NADPH generation for use in reductive biosyntheses (1). When rapid growth is possible, fewer secondary metabolites are produced, presumably because any excess nutrients are channeled into growth.

In a nutrient-limiting growth medium, cells are not dividing, and there is no demand for ribose 5-phosphate for the nucleotide precursors of DNA. Because the non-oxidative branch of the pentose phosphate pathway is controlled primarily by the availability of substrate, the ribose 5-phosphate (produced via the oxidative branch of the pentose pathway) is recycled into glucose 6-phosphate by enzymes of the gluconeogenic pathway. But, along with the increase in secondary metabolite production, there is also an increased demand for NADPH (for reducing power of biosynthesis). For this purpose, ribose 5-phosphate can be converted into pyruvate. Fructose 6-phosphate and glyceraldehyde 3-phosphate derived from ribose 5-phosphate enter the glycolytic pathway rather than reverting to glucose 6-phosphate. In this mode, NADPH and ATP are generated in large amounts, and 5 of 6 carbons of glucose 6-phosphate emerge in pyruvate. Pyruvate formed in these reactions can then be oxidized (via the TCA cycle and the electron transport chain) to generate more ATP, or it can be used as a building block in a variety of biosynthesis, including the production of natural products. Within the TCA cycle two key biosynthetic precursors, oxaloacetate and α -ketoglutarate, are specifically drawn off for biosyntheses, and must be replenished by pyruvate.

We isolated a bacterium, #J292/97, from a homogenized tissue sample of an American tube-dwelling anemone (*Cerianthopsis americanus*). Shake flask fermentations (100 ml) were

Table I

Induction of antimicrobial activity by various media

Media	Zone of inhibition (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
Marine Broth (MB)	0	0–0.3
MB diluted 1:9	3	8
MB diluted 1:9	4	13
0.1% α -ketoglutarate		
MB diluted 1:9	0	0
0.1% β -ketoglutarate		
MB diluted 1:9	1	0–0.3
0.1% glucose		
MB diluted 1:9	1	4
0.1% oxaloacetate		

performed in the following media: (A) Difco Marine Broth (MB) 2216; (B) MB (diluted 1:9 with filtered seawater); (C) MB (1:9) supplemented with α -ketoglutarate (0.1%); (D) MB (1:9) supplemented with β -ketoglutarate (0.1%); (E) MB (1:9) supplemented with glucose (0.1%); (F) MB (1:9) supplemented with oxaloacetate (0.1%). In preparing each medium, the pH was adjusted to 7.2. The shaking conditions were maintained at 220 rpm to ensure consistent aeration of fermentation flasks. After 72 h of fermentation, secondary metabolites were extracted with n-butanol and TLC was performed.

Vertical lanes were cut from the TLC plates from each extract and then exposed to a lawn of test bacterium: gram positive (*S. aureus*) and gram negative (*E. coli*). Diffusion of any antimicrobial compounds into the agar creates a zone of inhibition of bacterial growth. The size of this zone of inhibition indicates the strength of the compound's antimicrobial activity. These antimicrobial diffusion assays revealed an induction of an antimicrobial compound produced by J292/97 under nutrient-limited conditions (MB 1:9), as well as in medium supplemented with α -ketoglutarate (Table I). Neither β -ketoglutarate nor glucose showed induction.

The observation that the antimicrobial activity evident in nutrient-limited medium is amplified when supplemented with a TCA cycle intermediate seems to support our experimental model; the bioactive compound may be an intermediate drawn off the TCA cycle. Most of these intermediates for biosynthesis are derived from oxaloacetate and α -ketoglutarate; but MB (1:9) supplemented with oxaloacetate had no antimicrobial activity. Therefore, the α -ketoglutarate is being utilized either specifically in the TCA cycle pathway, or indirectly as a 5C organic acid.

Although medium supplemented with α -ketoglutarate induced antibiosis against both gram-positive and gram-negative test organisms, β -ketoglutarate as a supplement was inactive against both test organisms. We conclude that there may be a chiral specificity for the 5C compound, α -ketoglutarate, for the observed antimicrobial stimulation.

Low molecular weight carboxylic acids are produced and consumed during the TCA cycle and glycolysis, so these compounds are ubiquitous in marine organisms. Moreover, phytoplankton release a significant fraction of their fixed carbon into seawater as low molecular weight carboxylic acids; e.g., oxaloacetate and α -ketoglutarate (2). Therefore, these classes of organic compounds are usually present in natural seawater, and may be used by marine microorganisms as an important reser-

voir for necessary metabolic activities. Incomplete oxidations are a very interesting property of microorganisms whose importance for the production of chemical compounds will increase as the importance of secondary metabolites produced by marine microorganisms increases in the future. We are continuing to develop new culture methods in an effort to scrutinize the potential utility of this marine-based genetic resource.

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Antimicrobial Activity in the Microbial Community of the Accessory Nidamental Gland and Egg Cases of *Loligo pealei* (Cephalopoda: Loliginidae)

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In sexually mature female cephalopods, the accessory nidamental gland (ANG) harbors a dense bacterial community (1, 2). Relatively little work has been directed toward understanding the functions of the microbial community associated with this gland. Because of its close association to the nidamental gland and oviduct, the ANG has long been thought to play a role in protecting the eggs by coating them with symbiotic bacteria to ward off pathogens or predators. *Loligo pealei*, like many other cephalopods species, fixes its eggs in shallow water, in a substrate commonly called "egg-pom," forming a large communal aggregate. There is no parental guard during their long gestation period (45 days). The eggs are remarkably resistant to pathogens and predators. The presence of bacteria in association with egg jelly membranes has been observed in other cephalopods (3); however, no physiological role has been described for these bacteria. A preliminary experiment has shown that a butanol-ANG-extract could inhibit the growth of marine bacterial pathogens (*Vibrio anguillarum* and *Streptomyces griseus*).

In the present study, we sought to determine whether the ANG isolates produce antimicrobial compounds. We have also isolated bacteria from the internal egg jelly membranes to test their role in chemically defending the eggs. These data could suggest a role for ANG bacteria in the development of a complex microbe-host interaction.

Recently, at the Bay Paul Center for Comparative Molecular and Evolution at the Marine Biological Laboratory, we sequenced the 16S rDNA of the bacteria isolated from the ANG of *Loligo pealei*. *Alteromonas* sp. (Str. Ro1), *Shewanella* sp. (Str. Sq2), and *Roseobacter* sp. (Str. 303) were considered the representative ANG bacteria because of their predominance and occurrence in all ANG analyzed.

We tested the three representative ANG isolates (Str. Ro1, Str. Sq2, and Str. 303), as well as new isolates from the jelly membranes of the *Loligo pealei* egg cases. The specimens were collected by the Marine Resource Center at the Marine Biological Laboratory in June and July, 1997. Samples were taken from two egg cases, and all assays were performed in duplicate. The external outer layers of the egg cases were removed aseptically, and the egg cases were sterilized by immersing them in 75% ethanol for 1 min and washing them three times in sterile 75% seawater (SSW). The jelly membranes were removed from three successive levels toward the interior matrix, homogenized in 1 ml of 75% SSW, and plated by serial dilution (from 10^{-2} to 10^{-6}) onto three media: N1 marine agar 2216 (Difco), the control medium (used previously for isolating the ANG bacteria); N2 diluted (1:9) marine agar 2216 supplemented with 1% nidamental gland extract; and N3 diluted (1:9) marine agar 2216 supplemented with 0.1% of egg case extract. All cultures were incubated at room temperature and 4°C for 1–2 weeks.

Phenotypic analyses included colony and cellular morphology, flagella staining, oxidase and catalase activities, gram staining, salt requirement, proteolytic activity, H_2S production, nitrate reduction, and anaerobic growth (Bergey's Manual). Restriction fragment length polymorphism (RFLP) analysis was performed on the 16S rDNA of each strain, using four tetrameric restriction enzymes, *Hae*III, *Hin*PI, *Msp*I, *Rsa*I. The antimicrobial activity of the isolates was studied with standard inhibition diffusion assays, using a top layer of *Escherichia coli* as a gram negative, *Staphylococcus aureus* and *Streptomyces griseus* as gram positive, *Vibrio anguillarum* and *Aeromonas salmonicida* as marine pathogens, and *Laegidinium myophilum* as a common marine fungal pathogen (5). The antimicrobial compound was butanol-extracted from 100 ml marine broth 2216 (Difco) cul-

Table 1

Antimicrobial activity of accessory mandibular gland (ANG), egg cases, and bacterial isolates from *Loligo pealei*

	ANG	Egg cases	Str. Sq2 #1E, #2E	Str. Ro1 #3E, #4E	Str. 303 #5E, #6E, #8E, #9E	#7E
<i>Escherichia coli</i>				+	—	—
<i>Staphylococcus aureus</i>		—	±		—	++
<i>Sinclairomyces griseus</i>	++			++		+++
<i>Vibrio anguillarum</i>	+		+	+		+++
<i>Aeromonas salmonicida</i>	+	—	+	—	—	++
<i>Laegidialium myophilum</i>	nd	nd	+	+++		nd

— = negative; ± = slight activity (<0.5 mm); + = weak activity (<2.5 mm); ++ = good activity (2.5–5 mm); +++ = strong activity (5–15 mm); nd = not determined.

ture supernatants after 4 days of fermentation at room temperature, and concentrated by evaporation under nitrogen flux. Butanol-extracted homogenate from both fresh ANG and egg case jelly membranes was also tested in all assays.

Phenotypic analysis showed nine types of colonies (#1E to #9E). RFLP grouped these colonies as follows: *Shewanella*-like, #1E and #2E; *Alteromonas*-like, #3E and #4E; *Roseobacter*-like, #5E and #6E. The other three colonies showed a different RFLP pattern. Results obtained from the antimicrobial activity assay are presented in the Table 1.

Considering that one or more antimicrobial metabolites could be induced under nutritional stress, we also tried a butanol extraction of nutrient-limited growth media, following methods described elsewhere (6). Only Ro1, grown on marine broth diluted (1:9) plus 0.1% ANG, inhibited growth of the marine fungus *L. myophilum* (up to 5 mm).

The antifungal activity from *Alteromonas* Str. Ro1 suggests that an activity similar to that previously demonstrated in the

eggs of shrimp (7) could also occur in the squid. Further studies are planned using more concentrated extracts and larger numbers of potential pathogens to better understand mechanisms of how the bacterial symbionts of the squid ANG could affect their microhabitat, competitors, and predators.

This study was supported by an MBL Bernard Davis summer research fellowship.

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Reference: *Biol. Bull.* 193: 276–279. (October, 1997)

Coral Bleaching on Johnston Atoll, Central Pacific Ocean

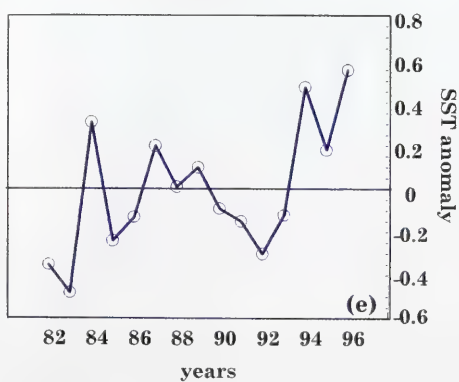
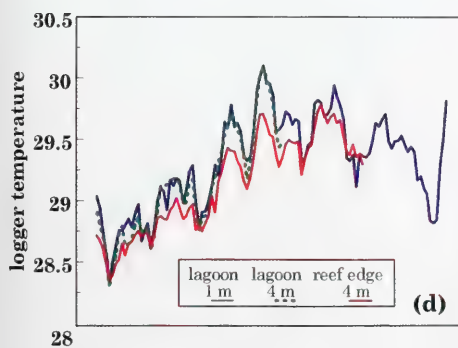
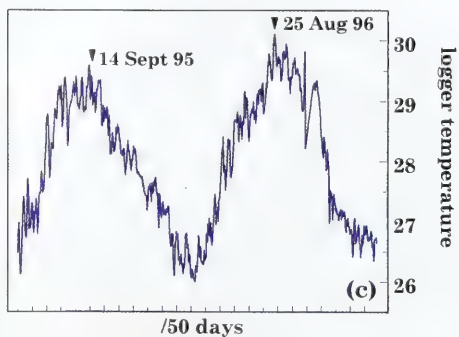
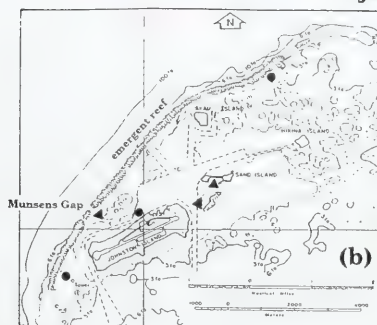
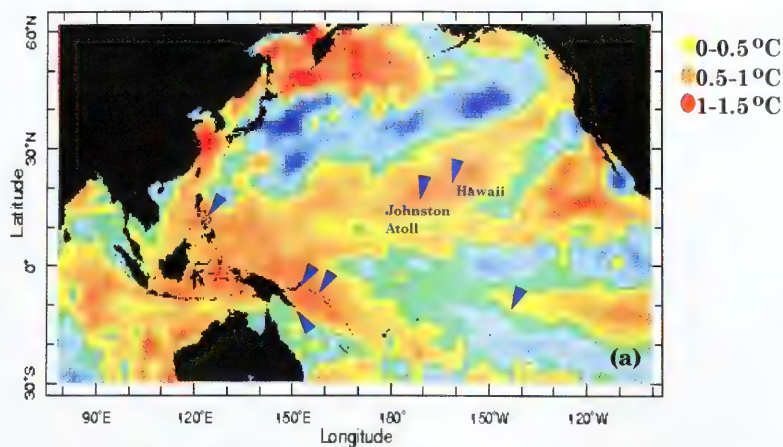
Anne L. Cohen, Phillip S. Lobel, and Gabrielle L. Tomasky (Boston University Marine Program, Woods Hole, Massachusetts 02543)

On 10 September 1996, extensive coral bleaching was noted on Johnston Atoll (JA), an isolated coral reef ecosystem in the central Pacific Ocean (16°N, 169°W). Between September 1996 and March 1997 we monitored the nature and extent of the bleaching, as well as the anomalous conditions of ocean temperature.

Coral "bleaching," or the loss of zooxanthellae and their photosynthetic pigments, is one of the first visible signs of thermal stress (1). The association between mass reef bleaching, and subsequent coral mortality, with elevated ocean temperatures is of concern in light of predicted global temperature increases over the next century (2). Mass bleaching is most

often associated with anomalous ocean temperatures during the warmest month of the year. A temperature increase of 1°–2°C above the historical mean summer maximum is considered necessary to induce coral bleaching in tropical and subtropical environments (1).

The 1996 JA bleaching event did not occur in isolation but appears to have been part of a global-scale bleaching episode that began in the western Caribbean and Gulf of Mexico in the summer of 1995 and was observed at several sites in the western and central Pacific the following year (3) (Fig. 1a). Satellite images indicate a basin-wide sea surface temperature anomaly (SSTA) of between 0.5°C and 1.5°C in September 1996 (4),



coincident with reports of coral bleaching on the Hawaiian Islands and on JA (Fig. 1a).

On JA, we examined six lagoonal and reef-edge sites to depths of 5 m (Fig. 1b) during three field excursions: 2–4 October, 21 October–5 November 1996, and 4–16 March 1997. Affected corals were tagged and photographed at one lagoonal site to monitor recovery rates. We distinguished between colonies that were fully bleached and those that showed partial bleaching, *i.e.*, bleaching confined to localized regions on a single colony. Our observations were as follows:

a. Bleaching was confined to corals in lagoonal sites (Fig. 1b). No bleaching occurred along the emergent reef with the exception of one bleached colony (*Pocillopora meandrina*) noted on the inside of the eastern reef edge.

b. Bleaching was species-specific. All *Montipora* spp. and *Pocillopora* spp. were affected, although the degree of bleaching of individual colonies varied from completely unaffected to partially bleached to complete loss of skeletal pigment. Bleaching was not observed in *Acropora cytherea*, the dominant coral species on JA.

c. Tissue loss from affected colonies did not occur during the first 3 weeks after the bleaching was noted, *i.e.*, 10 September–4 October 1996. Tissue loss was noted for several bleached *Pocillopora* colonies in late October. On the contrary, bleached *Montipora* colonies maintained living polyps for the duration of the bleaching event.

d. We estimated the areal extent of the bleaching in lagoonal sites to be from 15% to 20% between 0 to 5 m depth. Bleached corals were observed to a depth of 5 m.

e. By March 1997, 50% of the affected, tagged colonies had made a full recovery and regained pigment. Recovery was unrelated to the degree of bleaching experienced by individual colonies but was to some degree species-specific in that *Pocillopora* colonies without tissue were eventually overgrown by algae.

Daily temperatures at two lagoonal sites and one reef-edge site were recorded using temperature loggers (Branner Instruments) (Fig. 1b, c, d). The reef-edge site is a 6-m-deep channel (referred to as Munsens Gap; Fig. 1b) in the emergent reef structure. We regard temperatures recorded at this site to be representative of open-ocean mixed layer temperatures. *In-situ* temperature records in combination with IGOS NMC satellite-derived SSTs (4) (Fig. 1e) allow us to make the following observations:

a. Temperature loggers recorded a maximum summer lagoon temperature of 31.1°C on 25 August 1996, compared with a maximum summer temperature of 29.7°C in the previous year (14 September 1995) (Fig. 1c).

b. Average daily temperatures at the reef edge were 0.2°C lower than those recorded in the lagoon between 3 July and 21 October 1996. The maximum recorded SST at Munsens Gap was 29.8°C, and the maximum recorded difference between reef-edge and lagoonal sites was 0.4°C in late August 1996 (Fig. 1d).

c. Satellite-derived summer (JAS) SSTs for a $1^\circ \times 1^\circ$ grid square centered on 16.5°N, 169.5°W indicate an anomaly of 0.6°C in 1996 (compared with the historical mean since 1982) (Fig. 1e).

In timing, nature, and extent—including the species affected, tissue loss, and rate of recovery—the bleaching episode on JA was very similar to the one that was observed on the Hawaiian Islands in the same year (3; Paul Jokiel, University of Hawaii, pers. comm.) and that was predicted on the basis of laboratory manipulations of temperature (5, 6). However, whereas temperatures between 28°C and 29°C are sufficient to induce bleaching of *Montipora* spp. and *Pocillopora* spp. on Hawaii (1), congenics on JA appear tolerant of temperatures up to 29.8°C. Although the exact date when bleaching first occurred was not documented, the distribution of bleaching across the atoll, the timing of SST maxima in the lagoon, and the timing of the first observation of extensive bleaching lead us to conclude that the most sensitive coral species on JA have an upper thermal limit of about 30°C. This apparent difference in coral thermal tolerances between Hawaii and JA corresponds to differences in the maximum summer temperatures between these two sites.

Although *in situ* temperature data enable us to estimate the upper thermal limit of the affected JA coral species, the time series are too short to determine how high this limit is above normal summer SST maxima. The longer satellite-derived temperature record shows an anomaly of 0.6°C during the summer of 1996; according to field and laboratory observations, this anomaly is not high enough to induce coral bleaching by temperature alone (1, 5, 6). However, in assessing whether temperature was the sole cause of the JA coral bleaching event or whether other factors were involved, it is important to recognize the spatial resolution over which satellite-derived SSTs are averaged ($1^\circ \times 1^\circ$). Satellite temperatures are therefore representative of relatively large-scale open-ocean conditions and do not distinguish fine-scale temperature variability across the atoll. Our logger data indicate that small but important differences in SST occurred between sites on JA. SSTs in the lagoon, where bleaching occurred, were up to 0.4°C higher than those at the reef edge, where bleaching did not occur. Thus, we deduce that lagoonal temperatures were at least 1°C higher than the long-term ambient summer SST, which is in good agreement with that predicted to induce coral bleaching (1, 5, 6).

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Figure 1. Sea surface temperatures (SSTs) for the Pacific basin and for Johnston Atoll during the 1996 bleaching event. (a) Satellite-derived SST anomalies across the Pacific basin during September 1996 (Lamont-Doherty Earth Observatory Climate Data Catalog—ref 4). Arrows indicate sites where coral bleaching was reported to NOAA's Coral Health and Monitoring Program bleaching website during 1996 (3). (b) Map of Johnston Atoll showing monitored reef and lagoonal sites. Triangles denote sites where temperature loggers were deployed. (c) Daily SSTs at 1 m depth in Johnston Atoll lagoon: May 1995–February 1997. (d) Daily temperatures between 1 m and 5 m depth recorded at lagoonal and reef-edge sites on Johnston Atoll: 3 July 1996–31 October 1996. (e) Average summer (JAS) satellite-derived SSTs for a $1^\circ \times 1^\circ$ grid, centered at 16.5°N 16.5°W: 1982–1996 (4).

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PCB Contamination Relative to Age for a Pacific Damselfish, *Abudefduf sordidus* (Pomacentridae)

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Coral reef fishes grow rapidly during the first and second years of their life, attaining from 50% to 87% of their adult size (1, 2). During this period, growth is determined by various density-dependent and environmental factors (3, 4). Once a fish reaches maturity, somatic growth slows dramatically as energy is allocated for reproduction. The combination of these factors often produces fishes of different ages for a given size (5). Concentrations of polychlorinated biphenyls (PCBs) were highly variable in tissues of adult damselfish of about the same size collected from the same area at Johnston Atoll. These fish were aged to determine if there was a correlation with contaminant concentration. The hypothesis is that age equals exposure time, and therefore older fish are predicted to contain higher contaminant levels.

Adult damselfish, *Abudefduf sordidus* (Forsskal 1775) were collected to determine if PCBs sorbed to sediments were accumulating in their tissues. *A. sordidus* is a territorial spawner and a benthic omnivore, making it ideal for monitoring anthropogenic effects on coral reefs. *A. sordidus* gut contents include algae, benthic invertebrates, and sediments. Fish were collected from four areas in the lagoon of Johnston Atoll: (a) West Sand Island, with high (up to 389.0 µg/kg, or ppb) sediment PCB concentrations; (b) Buoy 14, with low (up to 0.5 ppb) PCB concentrations; (c) the former Herbicide Orange site, also with low (up to 7.2 ppb) PCB concentrations; and (d) East Sand Island, where additional fish were collected for aging only. The west end of Sand Island was the primary study site, where localized PCB contamination had been detected at concentrations exceeding ecological screening levels. The Buoy 14 and Herbicide Orange sites were used as background sites for PCB levels since no source, or substantial levels, of PCBs had been measured in the sediments there. However, other contaminants,

including dioxins and metals, also occur in sediments at the Herbicide Orange site. PCBs in sediments had a patchy distribution within and between sites (6). Sediments were composed of carbonate sand mixed with diatoms, dinoflagellates, and microinvertebrate infauna.

Fish ($n = 18$) were measured (standard length), weighed, and sexed; the otoliths were removed and weighed. Fish at each site were collected at the same time and were reproductively mature. Individual differences in recent reproductive output were not determined. Age was determined from thin, transverse sections of the sagittal otolith, mounted on a microscope slide and etched for 45 min with 2% hydrochloric acid. Otolith sections were viewed with transmitted light at 40× magnification. We found that discriminating opaque and translucent zones for aging was possible but difficult because the otoliths lacked a clear internal structure. Results from examination of 29 species of coral reef fishes, including three damselfishes, indicate that the alternating translucent/opaque patterns in the otoliths of coral reef fishes represent annual growth patterns (7). PCB and lipid content of the fish (without viscera) was analyzed by the Toxic Contaminant Research Laboratory, Wright State University, Ohio. Viscera were removed for other analyses. PCBs were analyzed using LRMS/HRGC and HRMS/HRGC (Modified EPA Method 8082 and Draft EPA Method 1668). Individual congeners of di- to deca-chlorinated PCBs were analyzed and summed to give total PCB concentration. Linear regression analysis was used to determine relationships between length, age, weight, and lipid content for all fish. Linear regression within sites was used to determine relationships between lipid and PCB concentration, and age and PCB concentration within sites because of the differing exposures at the sites.

There were significant relationships between length and weight ($r^2 = 0.913$, $P < 0.001$) and length and age ($r^2 = 0.767$, $P < 0.001$) for all fish. Ages varied from 5 to 9 years among fish at all sites, but individuals within a site were very close

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Table I

The age variability and PCB bioaccumulation in Abudcduf sordidus at four sites in the Johnston Atoll lagoon

Site	ID	SL (mm)	Weight (g)	Age (y)	PCB (ppb)	Lipid (%)	Sex	Otolith weight (g) ²
HO	8	135	166.6	7	33.6	4.2	F	0.0106
HO	9	135	167.9	7	191.0	2.6	NA	0.0103
HO	7	139	170.3	7	17.4	3.9	F	0.0094
HO	10	140	165.5	8	95.8	2.7	NA	0.0116
HO	6	145	201.7	7	59.2	4.7	M	0.0128
Mean (SD)		139 (4)	174.4 (15.4)	7 (0.4)	79.4 (69.0)	3.6 (0.9)		
WSI	6	145	202.2	8	767.0	5.1	M	0.0096
WSI	2	148	237.3	8	625.0	4.4	M	0.0148
WSI	1	150	NA	8	NA	NA	M	0.0088
WSI	3	152	228.1	8	1650.0	3.1	F	0.0130
WSI	4	152	253.2	8	1294.0	3.6	M	0.0206
WSI	7	155	226.0	9	288.0	2.4	F	0.0144
WSI	5	158	235.4	8	413.0	3.4	F	0.0146
Mean (SD)		151 (4)	230.4 (16.8)	8 (0.4)	839.5 (529.3)	3.7 (1.0)		
B14	3	123	124.6	6	86.2	2.1	F	0.0074
B14	2	127	128.5	7	30.0	2.6	F	0.0076
B14	1	138	170.4	7	6.6	4.0	F	0.0182
B14	5	140	176.1	8	10.9	4.6	F	0.0122
B14	7	140	205.3	8	9.8	4.8	M	0.0148
B14	6	143	198.3	7	53.2	8.3	M	0.0116
B14	4	148	192.6	9	10.2	2.6	M	0.0194
Mean (SD)		137 (9)	170.8 (32.5)	7 (1)	29.6 (30.0)	4.1 (2.1)		
ESI	1	120	109.3	5	NA	NA	NA	0.0062
ESI	5	125	120.9	6	NA	NA	NA	0.0084
ESI	4	140	163.7	7	NA	NA	M	0.0084
ESI	6	148	219.5	8	NA	NA	F	0.0147
ESI	3	160	242.2	9	NA	NA	F	0.0224
ESI	2	160	258.6	9	NA	NA	F	0.0160
Mean (SD)		142 (17)	185.7 (63.5)	7 (2)				

NA—Not analyzed.

¹ HO—Herbicide Orange site; WSI—West Sand Island site; B14—Buoy 14 site; ESI—East Sand Island site.² Average of left and right otoliths.

in age (Table I). There was no relationship between age and contaminant concentration for the West Sand Island and Herbicide Orange sites ($r^2 = 0.018$ and 0.261 , $P = 0.831$ and 0.301). The Buoy 14 site had a significant negative relationship between age and PCB concentration ($r^2 = 0.579$, $P = 0.047$). Contaminant concentration varied within age classes (Table I). PCB concentrations in 8-year-old ($n = 5$) fish from West Sand Island ranged from 413.0 to 1650.0 ppb. In addition, 7-year-old ($n = 4$) fish from the Herbicide Orange site had PCB concentrations ranging from 17.4 to 191.0 ppb. At the West Sand Island site the oldest fish (9 y) had the lowest PCB concentration; and at the Buoy 14 site the youngest fish (6 y) had the highest PCB concentration. There were no significant relationships between lipid content and length or weight ($r^2 = 0.012$ and 0.051 , $P = 0.661$ and 0.369). Additionally, there were no relationships between PCB concentration and lipid content at the three sites ($r^2 = 0.0003$, 0.001 and 0.573 , $P = 0.971$, 0.964 , and 0.139). There was no significant difference in PCB concentration be-

tween male and female fish at the Buoy 14 and West Sand Island sites (t test, $P = 0.92$ and 0.83). There were insufficient data to test differences at the Herbicide Orange site. Detection limits for the individual PCB congeners ranged from 0.001 to 0.054 ppb. PCBs detected in laboratory blanks ($n = 3$) ranged from 0.23 to 1.12 ppb.

Contaminant levels in fish tissues have been found to increase with trophic level and are often positively correlated with age and lipid content (8, 9). In this study, PCB tissue concentration was highly variable among individual fish with no correlation to size, age, or lipid content at two of the three sites. High PCB concentrations were in younger and smaller fish, while low concentrations were observed in older fish. In female pike (*Esox lucius*), contaminant concentrations were found to decrease with age due to the elimination of contaminants in the roe (10). The observation of this trend at one site in this study needs to be verified with larger samples sizes of fish with a greater size (age) distribution. Variable lipid and PCB concentrations in A.

sordidus could be the result of variable reproductive success. Male reproductive success of *A. sordidus* at Johnston Atoll varied from 1 to 20 clutches spawned in a 4-month period (6). Data on females were not available. Those males with high reproductive output would presumably have lower lipid and contaminant content due to the shedding of gametes and the high energetic cost of defending a nest site. Lipid content in whole fish is usually five to six times higher than in fillets, with a median of 40% less analyte found in fillets (11). Thus, even though the analysis of whole fish without viscera yields a somewhat lower contaminant estimate, the measurements are relative and comparison among individuals is still accurate.

The small variation in age by site compared to the large variation in PCB concentrations suggests that for this species of damselfish, lipid content and age or exposure period were not the main factors that determined PCB bioaccumulation. The pattern of PCB accumulation in *A. sordidus* at Johnston Atoll was independent of age (exposure duration), which is consistent with the patchy distribution of PCB contamination found in the sediments.

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assistance of Nina Shepherd and the staff of the Northeast Fisheries Science Center, Woods Hole Laboratory. We also thank Dr. T. O. Tiernan, Wright State University, for the tissue and sediment analyses.

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Comparative Settlement Age of Damselfish Larvae (*Plectroglyphidodon imparipennis*, Pomacentridae) from Hawaii and Johnston Atoll

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Most reef fishes have a planktonic larval phase with durations in the pelagic ocean habitat ranging from a few weeks to months. The pelagic larval duration (PLD) is an important factor associated with biogeographic distributions of reef fish populations (1, 2, 3, 4). The key question is this: to what degree are larvae passively advected and distributed by ocean currents, or are larvae somehow retained near natal habitats (5)? Schultz and Cowen (4) examined this issue of local retention *versus* long-distance transport of reef fish larvae to Bermuda. The PLDs for fish larvae newly settled on Bermuda reefs were compared with ocean current transport times. They found that the PLDs in their samples were similar to results obtained elsewhere in the fish's geographic range. Their conclusion was that fish larvae spawned in Bermuda were retained locally.

In this study, I compared the ages of settlement-stage larvae of the pomacentrid *Plectroglyphidodon imparipennis* (Vaillant and Sauvage, 1875) collected near Johnston Atoll and the island of Hawaii (Fig. 1a, b). If the source of recruitment of fishes to Johnston Atoll is the Hawaiian Islands, then PLDs of the larvae collected from Johnston Atoll should be longer than those of

the larvae collected near their point of origin in Hawaii. If larvae spawned locally on Johnston Atoll and Hawaii are retained near their natal habitats and recruitment is from the local population, then the PLDs from both locations should be very similar. Both Hawaii and Johnston Atoll have mesoscale eddies and other current patterns that may enhance local retention of ichthyoplankton (Fig. 1c) (5, 6, 7). Ocean circulation may also transport larvae across the distance from Hawaii to Johnston Atoll as shown by the drift tracks of current drogues (see below).

Johnston Atoll (16°45'N, 169°30'W) is an isolated coral reef habitat in the central Pacific Ocean. The nearest other reefs are Midway Atoll (about 460 nautical miles, nm, north), Oahu, Hawaii (about 717 nm northeast), and Palmyra Atoll, Line Islands (about 780 nm south by southeast). The fish fauna of Johnston Atoll is a mixture of Hawaiian and Line Islands species. About 301 species of reef fishes are found on Johnston Atoll: of these 53 are endemic to Hawaii and Johnston Atoll but are not found farther south, and 11 are indigenous to Johnston Atoll and the Line Islands but are not found farther north in Hawaii (8, 9). The question is whether these island faunas

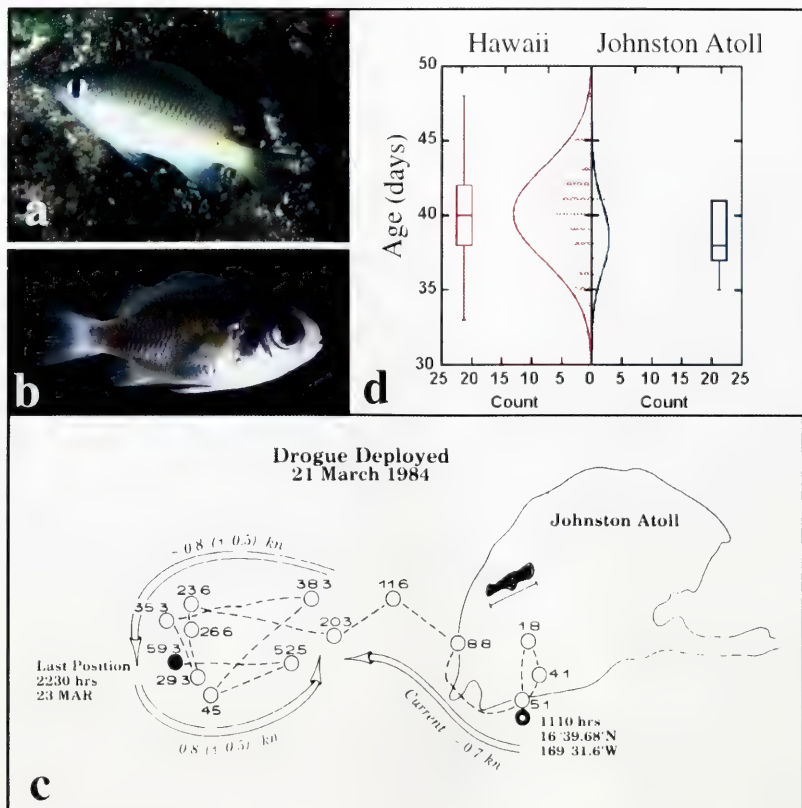


Figure 1. (a) Adult *Plectroglyphidodon imparipennis*, about 50 mm SL. (b) Settlement-stage larva, 14.1 mm SL. (c) Track of a current drogue; deployed March 1984, showing circulation in a cyclonic ocean eddy. Position numbers are hours elapsed since deployment. For distance scale, the island (in black) is 2 miles long. (d) Age distribution of settlement-stage larvae collected from Hawaii (n = 62) and Johnston Atoll (n = 10). A dual plot showing each datum; normal curve calculated using the sample mean and standard deviation is shown on the center vertical axis and a box plot displays sample median, quartiles, and outliers.

are continually exchanging larvae or if the biogeographic distributions of adult fishes resulted from rare instances of larval dispersal and transport. If the faunas were originally established by rare transport events, can island populations subsequently maintain themselves with local larval retention?

Plectroglyphidodon imparipennis is a small omnivore common on shallow reefs. The species has a very broad tropical distribution from East Africa to Hawaii, the Line, Marquesan, and Pitcairn Islands; north to the Ryuku and Bonin Islands; south to New Caledonia and Rapa; throughout Micronesia and the Indonesian archipelago (10). The color pattern of this species varies geographically.

Settlement stage larvae were collected by "nightlighting"; a bright light was suspended in the water near the surface. Larvae

attracted to the light were caught in a handnet and then preserved in 90% ethanol. Hawaiian larvae were collected from 20 February 1982 to 30 June 1982 within 5 nm of the coastline from Kailua-Kona to Keahou Bay, Hawaii. Johnston Atoll larvae were collected on 5 September 1983, also within 5 nm of reefs. Larval age was determined from otolith ring counts using the sagittae, the lapillae, or both. The highest ring count from a fish's otoliths was used as its age value (days). The current drogue (Fig. 1c) is the same type used by Lobel and Robinson (5).

The drift time for a passive larva to be transported from Hawaii to Johnston Atoll may range from 74 to 104 days (average 92 days). These data are from four satellite-tracked drogues during the period from February 1995 to March 1997 (11). For

an active larva, the transport time should be shorter than for a purely passive drifter. Larval fish can swim vigorously during the later developmental stages of post-flexion and settlement (12, 13, pers. obs.). Even assuming that active swimming accelerates the trek, larvae arriving on Johnston Atoll from Hawaii would still be expected to be at least a few weeks older than the local Hawaiian population. This assumes that there is a minimum developmental time until the larva reaches the settlement stage and that these fish are motivated to swim to the reef habitat at their earliest opportunity. It is unknown whether larvae orient and swim directionally over vast distances and how larvae find reefs.

The PLD was estimated from otolith ring counts, which are assumed to be daily growth increments as in other pomacentrids (1, 3). The average age of larvae collected from Hawaii ($n = 62$) was 40 days $\pm$ 3 SD (range 33 to 48) and from Johnston Atoll ($n = 10$) was 39 days $\pm$ 2 SD (range 35 to 41). The youngest settlement-stage larva collected was 33 days old and the oldest was 48 days old. Thus, it appears that *P. imparipennis* is competent for recruitment after about a month in the plankton but can delay metamorphosis for at least 2 weeks more. The ages of larvae collected from Hawaii were compared to larvae from Johnston Atoll (Fig. 1d) using a two-sample *t*-test (SYSTAT 6.0). Two tests were computed for comparing group means: the pooled variance *t*-test ($t = 1.528$, $df = 70$, $P = 0.131$) and the separate variance *t*-test ($t = 1.908$, $df = 15$, $P = 0.076$). The difference between the means was 1.597. The pooled test assumes that population variances are equal, whereas the separate variance test does not make this assumption. Results of both tests indicate no significant difference in the age of settlement-stage larvae collected from Hawaii and Johnston Atoll.

The conclusion is that the settlement stage *P. imparipennis* collected offshore Johnston Atoll were probably originally spawned there.

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Tidal River Riffle Habitats Support High Diversity and Abundance of Gammaridean Amphipods

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Gammaridean amphipods have an important role in estuarine food webs by serving as a trophic link from primary producers to higher-order consumers. Relatively little is known of their abundance, diversity, and distribution in estuaries of the Plum Island Sound region, northeastern Massachusetts. We studied amphipod populations along the brackish tidal portion of the Rowley River estuary, part of the Plum Island Sound estuarine system in Rowley, Massachusetts, to gain a better understanding of the environmental factors that influence amphipod ecology in this region.

A longitudinal survey was conducted during daytime low tide on 18 July 1997, to determine location and density of amphipods in hard and soft substrates. Rocks ranging in size from gravel to cobbles composed the hard substrate associated with low-tide riffles, and were variably covered with a turf of an attached chlorophyte alga (*Cladophora* sp.) and hydroids (*Cordylophora lacustris*). Soft substrate consisted of poorly sorted muds and sands associated with lentic low-tide water. Three hard-substrate and two soft-substrate sites were randomly sampled using a PVC collar (diam. = 3.5 cm) and an acrylic plastic core tube (diam. = 6.5 cm), respectively. After the longitudinal survey, one riffle habitat where the hard substrate was primarily cobble was selected for closer study. This site was sampled randomly at low tide on 23 July and 9 August 1997. The density of amphipods in three substrate cover types (bare rock, chlorophyte algal turf, and mixed hydroids and chlorophytes) was measured.

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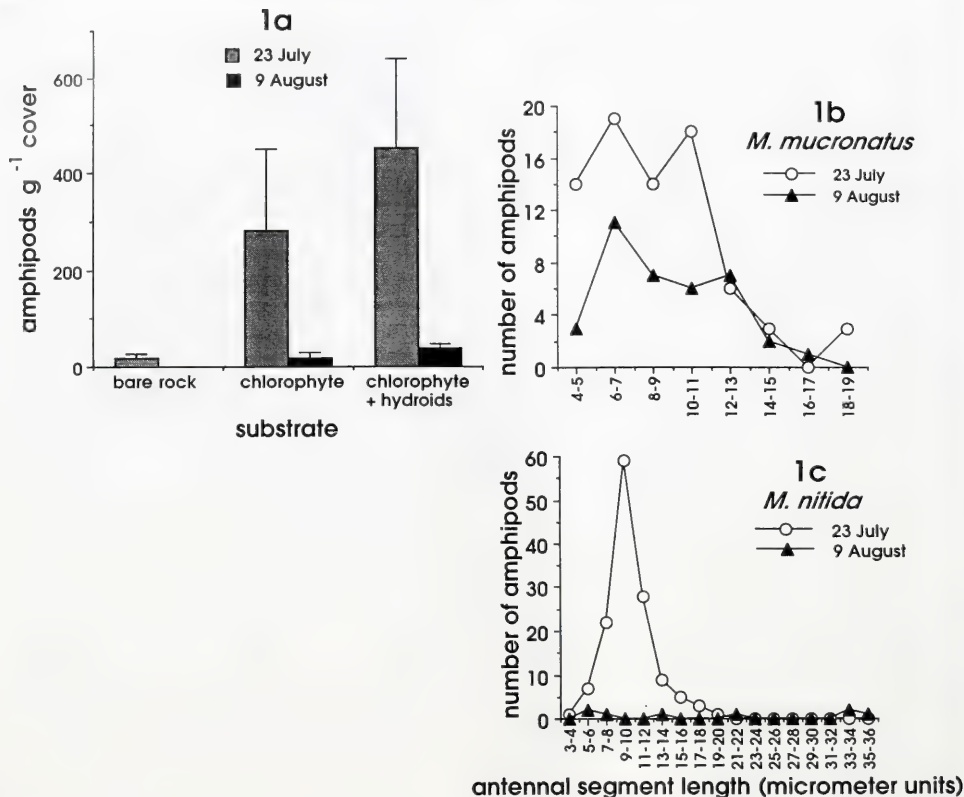


Figure 1. (a) Mean number of amphipods g⁻¹ of substrate cover, with standard errors, in a riffle habitat, Rowley River, summer 1997. (b and c) Size-frequency distributions for *Mucrogammarus mucronatus* and *Melita nitida* in the riffle habitat, each based on 9 samples for each date; data combined for chlorophyte and mixed-substrate cover types.

The four amphipod species found were *Corophium lacustre*, *Gammarus tigrinus*, *Mucrogammarus (Gammarus) mucronatus*, and *Melita nitida*. The latter two were abundant in the initial sampling and were selected for growth analysis. The relationship of segment 1, antenna 1 length to dry body weight was linear ($r^2 = 0.98$) for a range of sizes in these two species, allowing convenient determination of amphipod body size. The dry weight of the substrate cover for each of the samples was measured.

The density of amphipods in the hard substrates of the longitudinal study was significantly greater than that in the soft substrates (2.6 ± 0.33 vs. 0.02 ± 0.01 amphipods cm⁻², mean $\pm$ SE; $P < 0.025$, $F_{(1,10)} = 3.74$). The hard substrate held significantly more amphipod species than did the soft substrate (1.8 ± 0.2 vs. 0.5 ± 0.2 , mean $\pm$ SE number of species of amphipods per sample; $P < 0.001$, $F_{(1,10)} = 103.65$), even

allowing for the three-times greater sampling area of the soft-substrate core tube. Rocks covered with a mix of hydroids and chlorophytes made up most of the rock "population" (73%, compared to those with only chlorophytes [24%], and to bare rocks [3%]). The number of amphipods g⁻¹ substrate cover (Fig. 1a) was significantly higher in chlorophyte and mixed-cover types than on bare rock surfaces ($P < 0.01$, Tukey-Kramer method [1]), but was not significantly different between chlorophyte and mixed cover (overall P value for cover effect = 0.0001, 2-factorial ANOVA of cover type crossed with time, count data log-transformed). The number of amphipods g⁻¹ of cover declined significantly over 2 weeks ($P = 0.0002$). There was no significant interaction between cover type and time ($P = 0.94$). The dramatic decline in the abundance of amphipods (Fig. 1a) may be due to migration of individuals or to losses from predation. The size-frequency distribution of *M. mucrona-*

tus was relatively even and constant over time (Fig. 1b), suggesting continual recruitment and removal. In contrast, *M. nitida* showed a distinct cohort that was apparently short-lived in this habitat (Fig. 1c), which may indicate that this is an opportunistic species.

The abundance and diversity of amphipods in the Rowley River may be related to substrate complexity. In the primary hard-substrate study site the structurally complex substrates of chlorophytes and hydroids are common, and the amphipod abundance within them is high (Fig. 1a). The striking contrast between the density and diversity of amphipods in hard and soft substrates suggests that the hard-substrate habitat is supportive of amphipods with different behaviors and life histories (2, 3, 4; Fig. 1b, c). Important advantages conferred by these complex habitats, such as increased food availability (abundant

epiphytes and enhanced fine-particle deposition) and refuge from predators (5, 6), could explain the high amphipod densities observed during the study.

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Influence of Grazing and Nitrogen Loading on Benthic Microalgal Biomass in Estuaries of Waquoit Bay, Massachusetts

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Biomass of primary producers can be controlled by bottom-up (nutrient supply) and top-down (herbivory) mechanisms. Both processes are important in controlling benthic microalgae, a major component of primary productivity in intertidal and shallow subtidal marine sediments (1, 2, 3). In July 1997, we examined the relative importance of these processes in controlling the biomass of benthic microalgae in four estuaries of Waquoit Bay, Massachusetts, that are subject to different rates of nitrogen loading.

To assess the effect of nutrient supply, we measured the standing stocks of benthic microalgae in four estuaries of Waquoit Bay: Sage Lot Pond, Eel Pond, Quashnet River, and Childs River (4). The nitrogen loading rates from the watersheds to these estuaries were 64, 110, 520, and 624 kg N ha⁻¹ y⁻¹, respectively (WBLMER unpub. data). Study sites in the four estuaries had comparable salinities (27-32‰) and sediment porosities (30-39%). While faunal surveys were not conducted, the only major observable difference in faunal composition among sites was the lack of the mudsnail, *Ilyanassa obsoleta*, in Sage Lot Pond.

To measure the effect of grazers, we compared the standing stocks of microalgae in sediments of each estuary to standing stocks in sediments from which we excluded macroherbivores (including snails, shrimp, fish). To exclude grazers, we installed a set of seven translucent plastic cages (41 × 28 × 15 cm) into the bare soft sediments of each estuary at a mean depth of 1 m. The cages were driven 9 cm into the bottom sediments, so that

the head space within the cages was 6 cm. The tops of five cages per site were covered by 0.64 cm mesh. The remaining two cages were left uncovered as controls to determine possible cage effects as well as differences in unaccounted for estuary-specific variables. Three weeks after the cages were installed, we collected five cores (5.5 cm² in surface area × 2 cm in depth) from inside and outside each cage. The sediment samples were analyzed for chlorophyll *a* [a proxy for microalgal biomass (3, 5)] using the spectrophotometric method. Chlorophyll *a* was extracted using a solvent (45% acetone/45% methanol/10% deionized water; Joye, pers. comm.) modified from the standard method (6). The Lorenzen equation (7), modified to account for the new extraction solvent (Joye, pers. comm.), was used to correct for the presence of phaeopigments.

Chlorophyll *a* concentrations both inside and outside cages increased significantly with increasing nitrogen load ($r^2 = 0.999^*$, $r^2 = 0.965^*$; Fig. 1). This result highlights the importance of nutrient-related bottom-up effects in controlling benthic microalgal standing stocks, a result previously documented in estuarine (5) and in salt marsh sediments (2). Chlorophyll *a* concentrations inside of the cages were corrected for cage effects by subtracting the difference between mean chlorophyll concentrations inside and outside open cages of each estuary. The cages apparently reduced microalgal biomass, as chlorophyll *a* values inside of control cages were consistently lower relative to outside values: -19.6 ± 9.4 , -43.9 ± 6.3 , -51.2 ± 17.7 , -11.8 ± 37.3 mg m⁻² from low to high nitrogen load, respectively. We cannot explain the differences in cage effects across estuaries; differences may result because of estuary-specific differences in water flow regime or variability, or both, in

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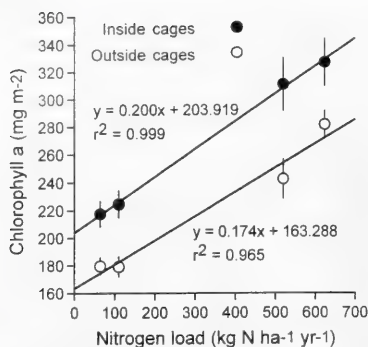


Figure 1. Sediment chlorophyll *a* concentration (mg m^{-2}) (mean $\pm$ standard error) measured inside and outside cages plotted versus nitrogen load ($\text{kg N ha}^{-1} \text{ yr}^{-1}$) in four estuaries of Waquoit Bay.

species composition and abundance of crabs and fish that consume microalgae or perturb sediments.

We assessed the magnitude of grazing by measuring the difference in chlorophyll *a* concentration inside and outside cages at the end of the study period. Grazers removed significant, nearly constant amounts of microalgal biomass (mean = $49.2 \pm 8.7 \text{ mg m}^{-2}$) irrespective of nitrogen load ($t = 890.5^*$, Fig. 1). Because of the cage effects, we can only tentatively conclude that grazers consume important amounts of microalgal chlorophyll, but our results document top-down control in agreement with previous findings in which removal of macroherbivores significantly increased microalgal biomass (8, 9).

Chlorophyll *a* increased with nitrogen load, but the amount of chlorophyll removed by grazer populations in each estuary remained constant. This suggests that the proportion of biomass grazed decreased as nitrogen load increased. This potential decrease in the significance of grazer impact as nitrogen load increases is similar to what was found in studies of amphipod grazing on macroalgal biomass within Waquoit Bay (10, Haux-

well, unpubl. data). These findings suggest that the relative importance of top-down and bottom-up controls in coastal marine systems may change as the systems become subject to nutrient enrichment, a phenomenon that has been documented in freshwater systems (11). As coastal ecosystems become increasingly eutrophic (12, 13), it may be that there will be a shift towards situations where top-down controls are overwhelmed by bottom-up controls. This is an important issue in coastal ecology, and our data are only suggestive. Further studies are needed to verify the results presented here.

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Control of Periphyton on *Zostera marina* by the Eastern Mudsnnail, *Ilyanassa obsoleta* (Say), in a Shallow Temperate Estuary

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The presence of epiphytes on eelgrass (*Zostera marina*) reduces irradiation reaching eelgrass blades and decreases eelgrass productivity (1, 2). Grazers (gastropods, amphipods, and isopods) may, however, increase eelgrass productivity (3, 4) by preferentially consuming epiphytes. To quantify grazer effects on epiphytes, we evaluated the consumption of periphyton (epiphytic microalgae, bacteria, microfauna, and detritus) on eelgrass leaves by the gastropod *Ilyanassa obsoleta* (Say) in Jchu Pond (average depth $\sim 1.2 \text{ m}$ below MLLW), an estuary of Waquoit Bay, Massachusetts.

We first related epiphyte biomass to snail density in the field. We then measured rates of periphyton removal by snails in the laboratory. Lastly, we extrapolated the laboratory grazing rates to field densities of snails to estimate the potential effect of *I. obsoleta* on eelgrass periphyton in the estuary.

We identified sites in the estuary containing relatively high, medium, and low snail densities (164, 100, and 52 snails m^{-2}) in early August 1997, by randomly sampling snail density using 12 quadrats (0.25 m^2) and 10 benthic grabs (0.0225 m^2). The

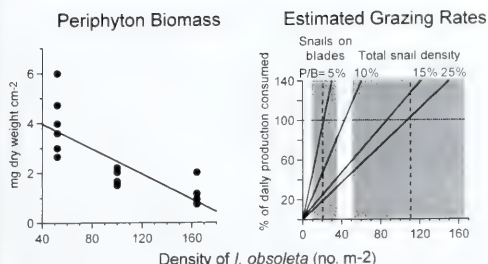


Figure 1. Left panel: Biomass of periphyton measured on *Zostera marina* leaves, plotted vs. density of *Ilyanassa obsoleta*. Points show values obtained for different blades within areas where snail density was measured. Periphyton biomass = $-0.025 \times \text{density of } I. obsoleta + 4.971$, $F = 28.586^{**}$, $r^2 = 0.632$, $P < 0.0001$ (Model I linear regression assuming the Berkson case [9]). Right panel: Estimated percentage of daily periphyton production consumed by *I. obsoleta* at varying densities, for eelgrass shoot density and epiphyte load measured in early August 1997. Possible production/biomass ratio (P/B) values are estimated as 5%–25% to represent the range of potential epiphytic production scenarios. Right-most shaded area corresponds to the range of total snail densities for which field periphyton biomass was measured (see left panel). Left-most shaded area represents snail densities on eelgrass leaves. Vertical reference lines denote mean snail densities. Horizontal reference line indicates the level of consumption above which net periphyton biomass is reduced by *I. obsoleta*.

proportion of total snail density on eelgrass blades vs. on sediment was determined for each quadrat. To measure epiphyte biomass from each site, five to seven leaf blades were harvested from randomly chosen shoots. To rule out variability in epiphyte biomass with leaf age, only the third blade in every shoot sampled was harvested. Periphyton was removed from both sides of each blade and weighed after drying overnight at 80°C.

Periphyton biomass decreased with increasing density of *I. obsoleta* (Fig. 1, left), suggesting control of periphyton biomass by this grazer. *I. obsoleta* density in the eelgrass bed ranged from 20 to 240 indiv. m⁻² (mean $\pm$ SE = 101.2 ± 23.3 indiv. m⁻²). Of the total density of snails in each quadrat, $21\% \pm 7\%$ (mean $\pm$ SE) were on eelgrass blades.

To measure periphyton grazing rates by *I. obsoleta*, we enclosed individual snails with eelgrass leaves colonized with periphyton. These experiments were run in clear plastic cages (15 cm $\times$ 15 cm $\times$ 3.5 cm) in a laboratory running seawater system. Initial periphyton biomass on 10-cm sections of blade was quantified by scraping periphyton from one randomly selected side of the blade and weighing it after drying at 80°C overnight. Use of periphyton from only one side of a blade to calculate the total epiphyte biomass per blade was validated by comparing the biomass on internal vs. external faces of eighteen 10-cm blade sections from different leaves. The mean epiphyte biomass did not differ between the two faces (t -test, $P = 0.529$), showing that the initial values of epiphyte biomass are similar for either side of a blade. To run the experiments, a section of blade was pinned scraped-side down to the bottom of each cage. Snails previously acclimated in the laboratory for 1–2 d with an excess of periphyton food (to simulate field conditions) were then placed in each cage (36 replicates) and allowed to graze for 24 h. Final epiphyte

biomass was obtained by scraping periphyton from the side of each leaf section that was available to the snail. *I. obsoleta* removed 11.3 ± 0.8 mg dry weight of periphyton day⁻¹ indiv.⁻¹ (mean $\pm$ SE) from eelgrass blades, which corresponds to $58.9\% \pm 5.8\%$ of the periphyton biomass on each blade section.

We extrapolated our laboratory measurements to field densities of snails to assess the possible effect of grazing by *I. obsoleta* on the estimated epiphytic production in Jehu Pond. Using our measured mean consumption rate, field measurements of mean eelgrass shoot density (127.3 ± 29.2 shoots m⁻² [unpubl. data]) and epiphyte load (0.0318 ± 0.0110 g shoot⁻¹ [unpubl. data]) and compiled literature estimates of the turnover rate of *Z. marina* periphyton (production:biomass ratio P/B = 5%–25% [5, 6]), we calculated the percentage of daily periphyton production (DPP) potentially consumed by varying densities of *I. obsoleta* (Fig. 1, right). The right-most shaded reference area in Figure 1 indicates the range of total snail densities for which field periphyton biomass was measured (in Fig. 1, left), and represents an upper limit on the ability of this grazer to consume periphyton. The left-most shaded area represents snail densities found on eelgrass leaves during sampling, used as a lower limit on our consumption estimate. If one considers the range of total snail densities, *I. obsoleta* could consume from 50% to >100% of DPP, depending on the actual P/B value. Using the densities of snails found on leaves, *I. obsoleta* could consume >100% of DPP, but only at low P/B values (~5%). These estimates suggest that *I. obsoleta* may be a control on periphyton biomass at certain snail densities, and at P/B values that occur in the field.

I. obsoleta is an important consumer of *Z. marina* epiphytes. By consuming significant epiphyte biomass, *I. obsoleta* may also influence eelgrass itself, as grazing rates similar to ones obtained here have resulted in increased eelgrass productivity in laboratory and mesocosm studies (7, 8). Periphyton may be a significant food source in this seagrass system; over 100% of daily production can potentially be consumed in sites where densities of *I. obsoleta* on eelgrass blades are high. Therefore, *I. obsoleta* can be an agent establishing top-down control of epiphytes as well as an important mechanism that influences eelgrass productivity and survivorship.

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The Importance of Access to Salt-Marsh Surface to Short-term Growth of *Fundulus heteroclitus* in a New England Salt Marsh

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The mummichog, *Fundulus heteroclitus*, is one of the most abundant fishes in Atlantic coast estuaries (1). Studies in Delaware (2) and Georgia (3) have shown the flooded salt-marsh surface to be an important foraging habitat, where small crustaceans and annelids are the primary prey. Pools on the surface

of New England salt marshes are surrounded by either *Spartina alterniflora*, in the low marsh, or *S. patens*, in the high marsh. The presence of *S. alterniflora* indicates a high frequency of flooding, whereas *S. patens* indicates a low frequency. Our hypotheses were (a) access to the marsh surface during marsh flooding enhances mummichog growth, and (b) the growth stimulus is greater in the more-frequently-flooded low marsh.

To test these hypotheses, we conducted field experiments in which we enclosed fish in cages that either allowed or denied access to areas of high or low marsh. We used two low-marsh pools and two high-marsh pools of the Rowley River salt marsh, northern Massachusetts. The pools were soft-bottomed, relatively small (<70 m²), and free of submerged aquatic vegetation. During the study period (24 July to 8 August 1997), water temperature fluctuated from 27°C (day) to 19°C (night) in all pools. Two cages were constructed of rigid 6.4-mm-mesh nylon netting and placed in each pool. The "no access" cage enclosed 1 m² of pool area, while the "access" cage enclosed 1 m² of pool area and 2 m² of adjacent marsh surface. Specimens of *Fundulus heteroclitus* were collected by seine, and total length (TL, ± 0.1 mm) and weight (± 0.01 g) measured. Eight groups of 10 fish were marked subcutaneously with a unique color of acrylic paint (4). Ten marked fish and 15 unmarked fish were placed in each cage for 2 weeks. After 1 week, a continuous temperature logger was deployed into each pool to monitor water temperature and tidal flooding. Flooding would cause an appreciable decrease in temperature (5) because the temperature of the flooding water was considerably lower than that of the water in the pools. At the end of the 2-week period, fish in the cages were recaptured in baited minnow traps, measured, and weighed.

Initial mean size of fish was 1.52 g (52.3 mm TL) and ranged from 0.84 g (47.2 mm) to 2.39 g (60.1 mm) (Fig. 1, top). Sixty-eight percent of the 200 fish introduced into the cages were recaptured. The marked fish were recaptured only in the cages in which they were originally placed, never outside the cages, indicating that the fish remained in the cage for the duration of the experiment. Final mean size was 1.59 g (53.0 mm) and ranged from 0.97 g (48.0 mm) to 2.97 g (62.6 mm) (Fig. 1). High tides flooded the low marsh on about half of the study days and the high marsh on less than half of the study days.

A two-factor [marsh level (high, low); access (yes, no)] nested (treatments within pools) repeated measures (initial and final means of treatments) analysis of variance was used to

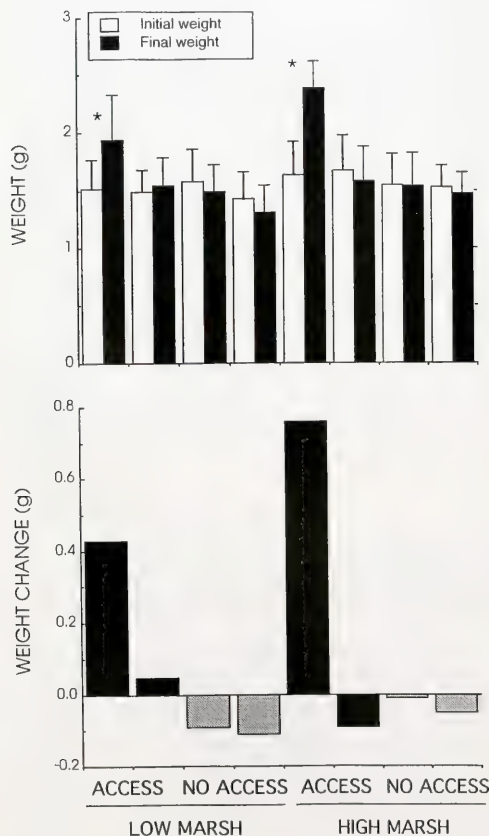


Figure 1. Top: Mean initial and final weights (g $\pm$ standard deviation) for each cage. Asterisks denote significantly different (t-test, $P < 0.01$) initial and final means. Bottom: Mean weight (g) change (final minus initial) for each cage.

assess the effects of marsh level and access on changes in either weight or length. The results indicated that marsh level had no effect (weight, $P = 0.20$; length, $P = 0.17$), and marsh access has a slight influence (weight, $P = 0.06$; length, $P = 0.06$), on mummichog growth. If growth increments are normalized by the total cage area and the amount of time access to marsh surface was possible (~ 1 wk), the effect of marsh access is decreased by only about $1/3$, discounting the influence of cage area alone on growth.

Two core samples taken from the marsh surface (33 cm²) indicated greater invertebrate abundance and species diversity in the high marsh (73 individuals; 6 species) than in the low marsh (27 individuals; 4 species). However, the more densely packed blades of *S. patens* compared to *S. alterniflora* may restrict foraging (5) enough to negate any potential nutritional gains from the more abundant and diverse prey of the high marsh compared to the low. Furthermore, since *F. heteroclitus* has been known to influence the abundance and distribution of its prey (6), it is possible that foraging by this species is directly responsible for the low prey abundance in the low marsh. These factors could account for the failure of marsh level to play a role in the growth of fish with access to the marsh surface. The data suggests that fish allowed access to any marsh surface will

have higher growth rates than those that are denied access. Although this study captured the essence of the tidal regimes on the marsh surface, its duration may have been too brief to permit enough foraging to affect growth strongly. It is also possible that mummichogs are less dependent on marsh surfaces in New England than are their conspecifics in the more southern ranges of the species distribution.

This work was funded by NSF Research Experience for Undergraduates and the Plum Island Sound LMER Program. We thank the Essex County Greenbelt Association for use of their marsh.

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Growth Rates and Abundance of *Fundulus heteroclitus* in Estuaries Subject to Different Land-Derived Nitrogen Loads in Waquoit Bay, Massachusetts

Alison Whitman¹, Joanna Tober, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

Increased land-derived nitrogen loading in estuaries of Waquoit Bay, Massachusetts, increases phytoplankton and macroalgal production and biomass (1). The enhanced primary productivity then prompts increases in secondary production including fish abundance (2, 3, 4). To confirm the effect of nitrogen loads on fish abundance, and to determine whether the growth rates of fish are also increased, we measured abundance and growth rates of *Fundulus heteroclitus* in populations found in estuaries subject to different rates of land-derived nitrogen inputs. *F. heteroclitus* is the most abundant fish species in the near-shore waters of Cape Cod. This is an omnivorous species (5, 6), and its populations have relatively small home ranges (7). This reduced mobility makes it feasible to compare populations found in different estuaries, even when the estuaries are near each other.

To assess the relationship of fish growth rate versus abundance, and the possible link of nitrogen load on that relationship, we sampled in four estuaries that receive different nitrogen loads: Childs River (624 kg N ha⁻¹y⁻¹), Quashnet River (520 kg N ha⁻¹y⁻¹), Eel Pond (110 kg N ha⁻¹y⁻¹), and Sage Lot

Pond (64 kg N ha⁻¹y⁻¹) (unpub. data, WBLMER). At five sites in each estuary we towed a 10-m seine for 10 m. After each tow, we counted and measured the fish caught. Tows were conducted on a monthly basis from June through August, and additional tows were made to ensure that we had enough fish at each size class for the growth rate studies.

We measured growth rates of all the fish in the sample. Standard lengths of fish were measured, and otoliths were extracted and mounted on slides using a liquid cover slip. We determined the age of each fish by counting yearly growth rings on the otoliths under a dissecting microscope (8, 9). To calculate growth rates for fish of all age classes and for fish in age class zero, we plotted ages of the fish versus their standard lengths. We did this for each estuary, pooling the data from all sites and all months, and used the slope of each line as the growth rate for that estuary.

On first principles of competition theory, growth rate of individual fish should decrease as the abundance of a fish population becomes greater, unless food supply increases in tandem with abundance (10). The growth rates of *F. heteroclitus* in the Waquoit Bay estuaries, in contrast to what we might have expected, increased as abundance increased (Fig. 1). The error in Figure 1 is not suitable for analysis of variance because it is based on

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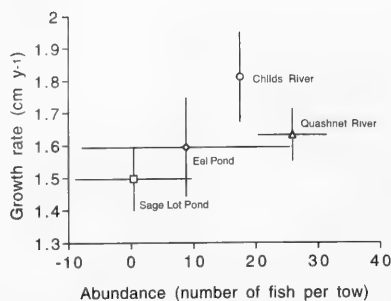


Figure 1. Growth rate (mean $\pm$ SE obtained from the regression of length versus age) of *F. heteroclitus* for all fish classes, versus total number of *F. heteroclitus* specimens caught per seine tow (mean $\pm$ SE) in five sites within the four different estuaries of Waquoit Bay.

internal pseudoreplicates (11). Instead, we used a non-parametric test of association, Kendalls τ , which showed that there was a significant association, at the 0.05 probability level, between growth rates and abundances in Figure 1.

The result that growth tends to increase with abundance suggests that some factor, perhaps food supply, increases in parallel with abundance of the fish. The order in which the estuaries appear in Figure 1 (Sage Lot Pond on the lower left, and Childs and Quashnet rivers on the top right) suggest that the estuaries that receive higher nitrogen loads from their watersheds might produce somewhat more fish that perhaps grow faster. Of course we cannot eliminate the possibility that other estuary-specific

features are also involved. If larger abundances of fish do not, indeed, restrict the abundance of the food supply available for the fish population, as implied by the positive slope of Figure 1, then we can speculate that in these estuaries the influence of top-down controls by fish might be overwhelmed by bottom-up controls related to nitrogen loading rates.

This research was supported by funds from an NSF Research Experience for Undergraduates grant. Special thanks to Kathy Lang and the rest of the staff at the National Marine Fisheries Service in Woods Hole for help with the otolith aging techniques and for the use of their equipment. We thank the unidentified reviewer for helpful comments on earlier versions of this paper.

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Effect of Salinity on the Fate of Inorganic Nitrogen in Sediments of the Parker River Estuary, Massachusetts

Summer Morlock (Duke University), David Taylor¹, Anne Giblin², Chuck Hopkinson², and Jane Tucker²

Sediments are a major site controlling the cycling and availability of nitrogen in estuaries (1). In sediments, inorganic nitrogen (i.e., ammonium, nitrate, nitrite) can diffuse to overlying water, be adsorbed onto sediments, or be denitrified (2). Previous studies have suggested that the fate of nitrogen in sediments may be influenced by salinity (3). High cation concentrations in salt water may decrease ammonium adsorption onto sediments by occupying cation exchange sites, increasing the relative amounts of free to exchangeable (adsorbed) ammonium in the sediments (3). Because primary productivity in many estuarine systems is nitrogen limited (4) and the salinity structure of estuaries can vary substantially over time, it is necessary to study the importance of this possible "salt effect."

We examined the effects of salinity on sediment nitrogen cycling, including the ratio of free to exchangeable ammonium in sediments, inorganic nitrogen fluxes across the sediment-water interface, and denitrification. Twenty sediment cores (6.5 cm in diameter and 12 cm deep) were collected from the upper reaches of the Parker River Estuary (PRE), Massachusetts, where salinity varies from 0 to 18 ppt annually (Fig. 1a). Overlying water in 10 cores was replaced with seawater (32 ppt). The remaining 10 cores were held at an ambient salinity (0.7 ppt). To estimate denitrification, half of the high-salinity and low-salinity cores were incubated with anoxic overlying water. Under this condition, nitrification and subsequent denitrification are halted; therefore total inorganic nitrogen accumulated in the sediments and the overlying water could be compared between oxic and anoxic treatments to estimate denitrification. This estimate assumes that rates of nitrogen remineralization are similar under oxic and anoxic conditions.

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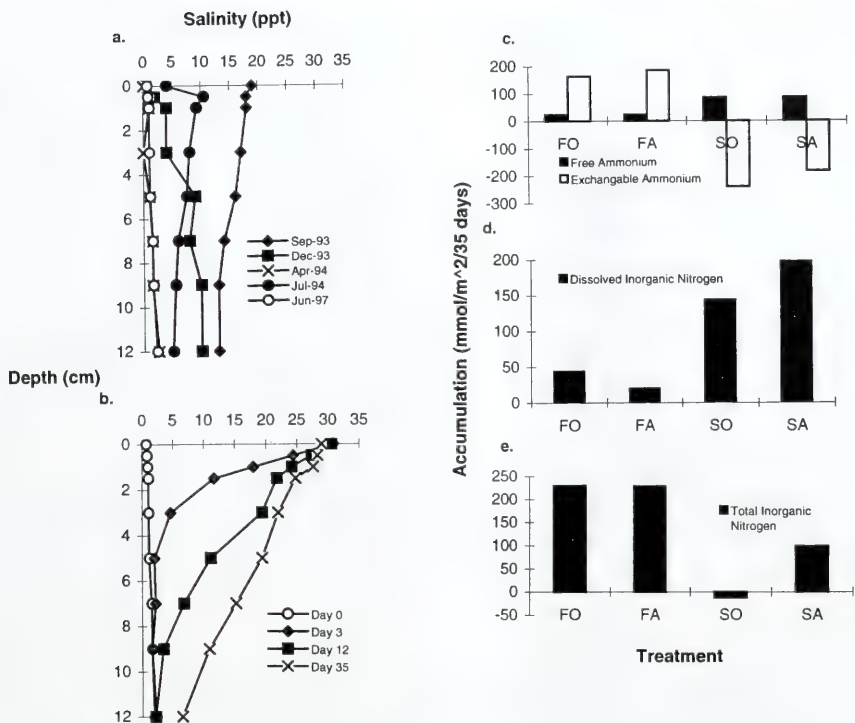


Figure 1. (a) Salinity profiles typical for the Parker River Estuary site over a year. Open symbol reflects the profile of the initial untreated core analyzed during this study. (b) Average salinity profiles for salt-water treatments during the experiment. (c) Accumulation or loss of free and exchangeable ammonium in sediments during the 35-day incubation. (d) Accumulation of dissolved inorganic nitrogen in the overlying water during the 35-day incubation. (e) Accumulation or loss of total inorganic nitrogen in sediments and overlying water during the 35-day incubation. Abbreviations: FO = fresh oxia, FA = fresh anoxic, SO = salt oxia, and SA = salt anoxic.

For all cores, the overlying water was changed every 3 to 7 days and sampled for ammonium and nitrate/nitrite. At intervals throughout the 35-day study, one core from each treatment was sectioned into 2-cm intervals (three untreated cores were analyzed on day 0 for baseline analysis). Free and exchangeable ammonium concentrations and chloride concentrations were analyzed in each sediment section. The concentrations of free ammonium, exchangeable ammonium, and dissolved inorganic nitrogen fluxes (ammonium and nitrate/nitrite) to the overlying water were individually summed at each sampling time, and then subtracted from their initial baseline concentrations. These results represented the total change in free and exchangeable ammonium in the sediments and the total amount of dissolved inorganic nitrogen (DIN) fluxed to the overlying water over the course of the study.

Throughout the experiment, the fresh-water chloride profiles remained similar to the initial profile, or Day 0 (Fig. 1b). The chloride profiles of the salt-water treatments indicated salt intru-

sion with depth over time (Fig. 1b). By the end of the investigation, there was a greater accumulation of free ammonium in the salt-water treatments than in the fresh-water treatments (Fig. 1c). In addition, the salt-water treatments experienced a net loss of exchangeable ammonium, while the fresh-water treatments gained exchangeable ammonium (Fig. 1c). Also, by the end of the study, the total amount of DIN released to the overlying water was much greater in the salt-water treatments (Fig. 1d). Summing fluxes to the overlying water and sediment stocks (Fig. 1c + Fig. 1d), the overall increase in total inorganic nitrogen was greater in fresh-water treatments than in salt-water treatments (Fig. 1e). The lower response in the salt-water anoxic treatment may be due to decreased metabolism produced by salinity shock at the outset of the experiment (4).

We were not able to perform statistical analyses on the summed data (Fig. 1c, d, and e) because only one core from each treatment was analyzed at each time point. However, for individual time points, the rate of DIN flux to the overlying

water was significantly greater in the salt-water treatments than in the fresh-water treatments ($P < .05$, t -test) (data not shown). Also, the ratio of free to exchangeable ammonium at the end of the study was significantly greater in the salt-water treatments ($P < .01$, t -test) (data not shown). This suggests that salt intrusion significantly affects nitrogen cycling in estuarine sediments and that differences between the fresh-water treatments and salt-water treatments in Figure 1c, d, and e are real.

There was no notable difference in the amount of total inorganic nitrogen between the fresh-water oxic treatments and the fresh-water anoxic treatments (Fig. 1e). There was a difference between the salt-water treatments: a net inorganic nitrogen gain in the anoxic sediments and a net loss in the oxic sediments (Fig. 1e). Although this difference could not be statistically evaluated, it appears that denitrification was more important in the salt-water treatments than in the fresh-water treatments. These results are contrary to those of Seitzinger *et al.* (5), who suggested that denitrification is greater in fresh water. Future study should include direct, repeated measurements of denitrification, to better understand differences in the effects of the variation of salinity on denitrification.

The results of this study suggest that salinity had a major influence on the distribution and fate of nitrogen remineralized in benthic sediments. The "salt effect" that we observed appeared to be an increase in the ratio of free to exchangeable

ammonium, which supports observations of Gardner *et al.* (3). In addition, a subsequent effect of increased salinity appeared to be an increased DIN flux to the overlying water. This result is consistent with previous observations in the Parker River Estuary where ammonium fluxes were unusually high (relative to sediment remineralization) when porewater experienced large increases in salinity during the summer. Therefore, it appears that this high ammonium flux represented not only current remineralization, but also ammonium originally stored on the sediment exchange complex.

This research was partially supported by grants from WHOI Sea Grant, the Plum Island Sound LMER, the Sweetwater Trust, and the NSF-REU Boston University Marine Program.

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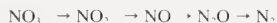
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Release of N_2 and N_2O from Salt-Marsh Sediments Subject to Different Land-Derived Nitrogen Loads

Rosalynn Y. Lee¹, Samantha B. Joye², Brian J. Roberts, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

Coastal systems receive the highest loading of anthropogenic nutrients in the world (1, 2). Denitrification (DNF), the microbial respiration of nitrate (NO_3^-) to gaseous end products, occurs in sediments of salt marshes fringing estuaries, and provides a nitrogen sink that reduces land-derived N loading to estuarine waters. The reduction process



can result in the release of nitrous oxide (N_2O), as well as nitrogen gas. This so-called incomplete DNF has global implications, because N_2O is a trace greenhouse gas that can consume stratospheric ozone.

We used the acetylene inhibition technique (3) to measure rates of DNF in three estuaries—Chilts River (CR), Quashnet River (QR), and Sage Lot Pond (SLP)—in the Waquoit Bay, Massachusetts, watershed. The estuaries receive N loads of 624,

520, and 64 kg N ha⁻¹ yr⁻¹, respectively, and hence provide the opportunity to see if DNF varies in salt marshes exposed to different regional-scale regimes of land-derived N inputs.

To assess whether the local availability of NO_3^- altered potential rates of DNF, different concentrations of NO_3^- (ranging from 0–5 mM) were added to three replicate bottles containing 2 ml of a slurry of sieved marsh sediment collected from the top 5 cm at each of three sites in each estuary, and 2 mM glucose amended site-specific water. The headspace was purged with helium, and each NO_3^- treatment was run in triplicate with and without acetylene (C_2H_2), which blocks the enzyme that reduces N_2O to N_2 . The N_2O produced in the presence of C_2H_2 represents the total potential DNF. The N_2 produced was calculated as the difference between total potential DNF and N_2O production in the absence of C_2H_2 . After a 5–6 h incubation, N_2O production was quantified using a Shimadzu gas chromatograph with an electron capture detector.

Rates of total potential DNF and N_2O production exhibited Michaelis-Menten saturation kinetics with increased NO_3^- additions (data not shown). The saturation rates (V_{max}) of both total potential DNF and N_2O production increased as the land-derived

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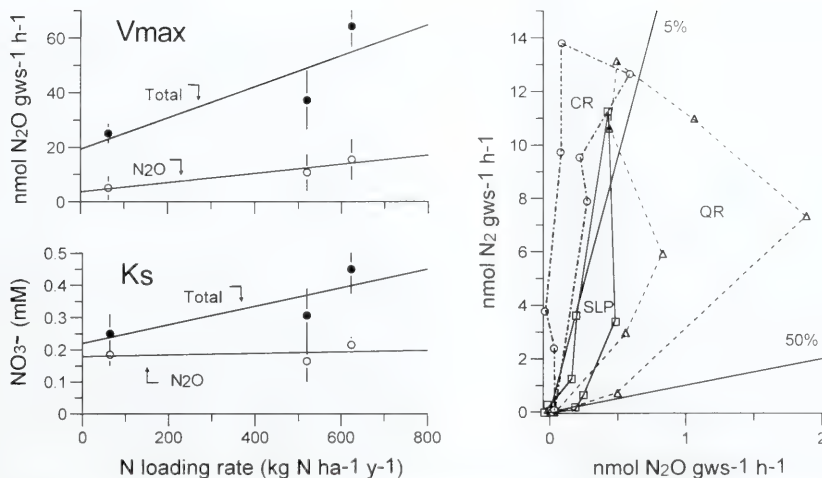


Figure 1. Top left: Relationship of saturation rates (V_{max}) of total potential DNF ($y = 0.057x + 19.299$, $r^2 = 0.715$) and N_2O production ($y = 0.017x + 3.669$, $r^2 = 0.915$) in fringing salt-marsh sediments, relative to N load. Bottom left: Relationship of half saturation constants (K_s) of total potential DNF ($y = 0.000x + 0.220$) and N_2O production ($y = 0.000x + 0.179$) to N load. Right: N_2 vs. N_2O production in Waquoit Bay estuaries—CR: Childs River, QR: Quashmet River, and SLP: Sage Lot Pond. Data includes only additions of 0–1 mM NO_3^- . Whole lines show where values might fall if ratios of N_2 to N_2O are 5% and 50%. Dashed lines circumscribe values for the three estuaries.

N load increased (Fig. 1, top left). The half saturation constant (K_s) from total potential DNF also increased with loading, whereas the K_s of N_2O production did not change (Fig. 1, bottom left).

The relative release of N_2 and N_2O from these salt marsh sediments changed with the amount of experimentally added NO_3^- and with the load of land-derived N (Fig. 1, right). We plotted N_2 and N_2O production only for measurements done at lower NO_3^- additions (<0.1 mM NO_3^-), emulating *in situ* concentrations (4). Previously reported measurements of $N_2O:N_2$ in marine and freshwater sediments are usually $<5\%$ (4). The N_2O and N_2 released from Waquoit salt-marsh sediments spanned a wide range. All the N_2O to N_2 ratios for CR (the most highly loaded estuary) were consistent with literature values, and fell below the 5% $N_2O:N_2$ line. Our ratios for the less loaded estuaries, QR and SLP, fell between 5% and 50%, indicating greater release of N_2O *in situ* relative to N_2 at lower N loads. This might mean that salt-marsh sediments exhibit greater variability in $N_2O:N_2$ released than do lake, river, and other coastal marine sediments (4). When inputs of groundwater increase NO_3^- supply and saturate rates of production (Fig. 1, top left), greater amounts of N_2O are released in estuaries with higher N loads, which agrees with other reports (5). The rate

of release of N_2O relative to N_2 may decrease as loading increases; the ratio in CR sediments is less than that of the other estuaries (Fig. 1, right). As loading increases, any local increases in NO_3^- concentrations could release more N_2O into the atmosphere, while supply of NO_3^- determined by regional loading regimes will lower N_2O produced by denitrification compared to N_2 release.

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Field Verification of Predictions of the Waquoit Bay Nitrogen Loading Model

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Eutrophication of coastal waters by increasing anthropogenic nutrient inputs is arguably the dominant agent of change to coastal ecosystems (1, 2). The Waquoit Bay Nitrogen Loading Model (WBNLM) was developed to estimate rates of nitrogen loading from watersheds to coastal receiving waters. WBNLM includes inputs from wastewater, fertilizer use, and atmospheric deposition, and defines losses within soil, vegetation, vadose zone, and aquifer (3). Here, we use WBNLM to estimate nitrogen loads to two subwatersheds of the Waquoit Bay estuary, Eel Pond and Head of the Bay (2), and to verify the model predictions by comparing the model estimates to field measure-

ments of nitrogen load. We also included unpublished WBNLM estimates and field data from other estuaries of the Waquoit Bay system in our comparisons.

The Waquoit Bay drainage basin is underlain by unconsolidated sandy sediments of glacial origin. Due to the high infiltration rate of this type of material, groundwater transports the bulk of the nutrients entering the estuaries (3). To measure the nitrogen load moving in groundwater from land to estuary, we first determined nitrogen concentrations in samples of groundwater (salinity < 0.5‰) at the seepage face of the aquifer. These samples were collected from stations around the periphery of the two estuaries at intervals of 25–100 m by using a drive-point piezometer about 3–5 m from the waterline at depths of 1 to 1.5 m. Water samples were filtered with a 0.47- μm pore-size membrane filter and acidified. Concentrations of total dissolved nitrogen (TDN) were measured with a LACHAT auto-analyzer using a method adopted from D'Elia *et al.* (4). For Eel Pond, we found (mean $\pm$ SE) concentrations of 157.9 ± 55.0 , 137.3 ± 35.9 , 93.1 ± 12.0 , 147.8 ± 69.9 , 141.0 ± 60.6 , and 165.6 ± 59.5 μM for TDN, respectively, for recharge areas 1, 2E, 2W, 3E, 3W, 4 (Fig. 1, left). For Head of the Bay, we measured TDN concentrations of 60.2 ± 6.8 μM .

To obtain a yearly loading rate, we multiplied the average concentrations of total dissolved nitrogen in the groundwater by an estimation of annual recharge to the receiving water (45% of total precipitation). The model estimates recharge without specifying where on the watershed deposition occurs (3). Land-use estimates for modeling of both watersheds were taken from Geographical Information Systems land-use maps of the Waquoit Bay watershed.

In model verification there is always the question of what spatial scale is most appropriate for model predictions. To answer this question, we ran WBNLM for entire watersheds and for smaller subdivisions called "recharge areas." These recharge areas all have different land-use mosaics, and therefore different nitrogen loads. For example, we subdivided the Eel

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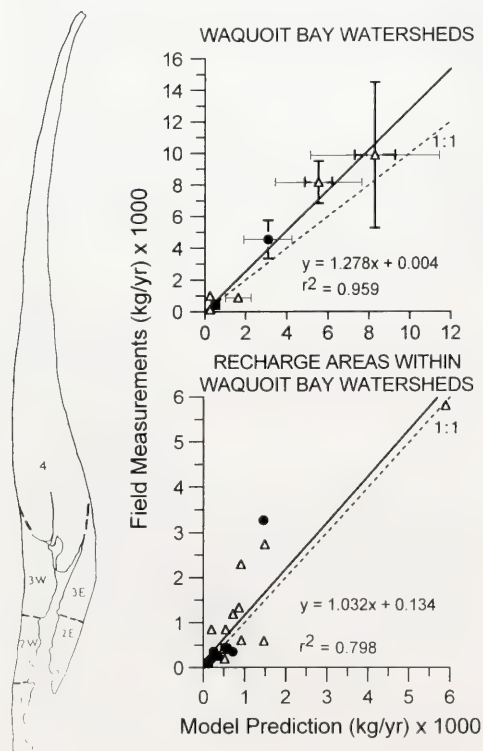


Figure 1. Left: Eel Pond watershed with recharge areas (1, 2 East, 2 West, 3 East, 3 West, and 4). Bottom right: Mean field measurements of nitrogen-loading rates for recharge areas within the Waquoit Bay subwatersheds plotted versus the mean model predictions. Top right: Mean field measurements of nitrogen-loading rates for subwatersheds of the Waquoit Bay estuary versus model predictions. Standard deviations are represented by thin error bars, and standard errors are represented by thick error bars. Both standard deviations and standard errors for the model were calculated using a bootstrap method (Collins *et al.*, unpubl. data). Field and model values for other Waquoit Bay watersheds were taken from unpublished data collected by the Waquoit Bay Land Margin Ecosystems Research project. These watersheds are identified in (3). Eel Pond (●), Head of the Bay (■), all other Waquoit Bay watersheds (△).

Pond subwatershed into six recharge areas on the basis of groundwater flow patterns (Fig. 1, left). We apportioned recharge to each in relation to the catchment area. The Head of the Bay subwatershed was not subdivided.

Predicted nitrogen loads to different estuaries of Waquoit Bay increased linearly in relation to estimates based on field measurements (Fig. 1, top right). The model slightly underestimated nitrogen loads within watersheds, and the underestimation increases as the nitrogen load increases. Within the range of nitrogen loads to Waquoit estuaries, the maximum percent deviation of the model estimates from the 1:1 line (Fig. 1, top right) is only 20%, and the deviation is within standard deviations calculated for the model estimates (2). Sensitivity analysis of the model, not included here, suggests that the differences between model estimates and observed field values may result from an overestimation of losses in the vadose zone or in the aquifer, or from overestimation of the amount of labile relative to refractory dissolved organic nitrogen entering the system.

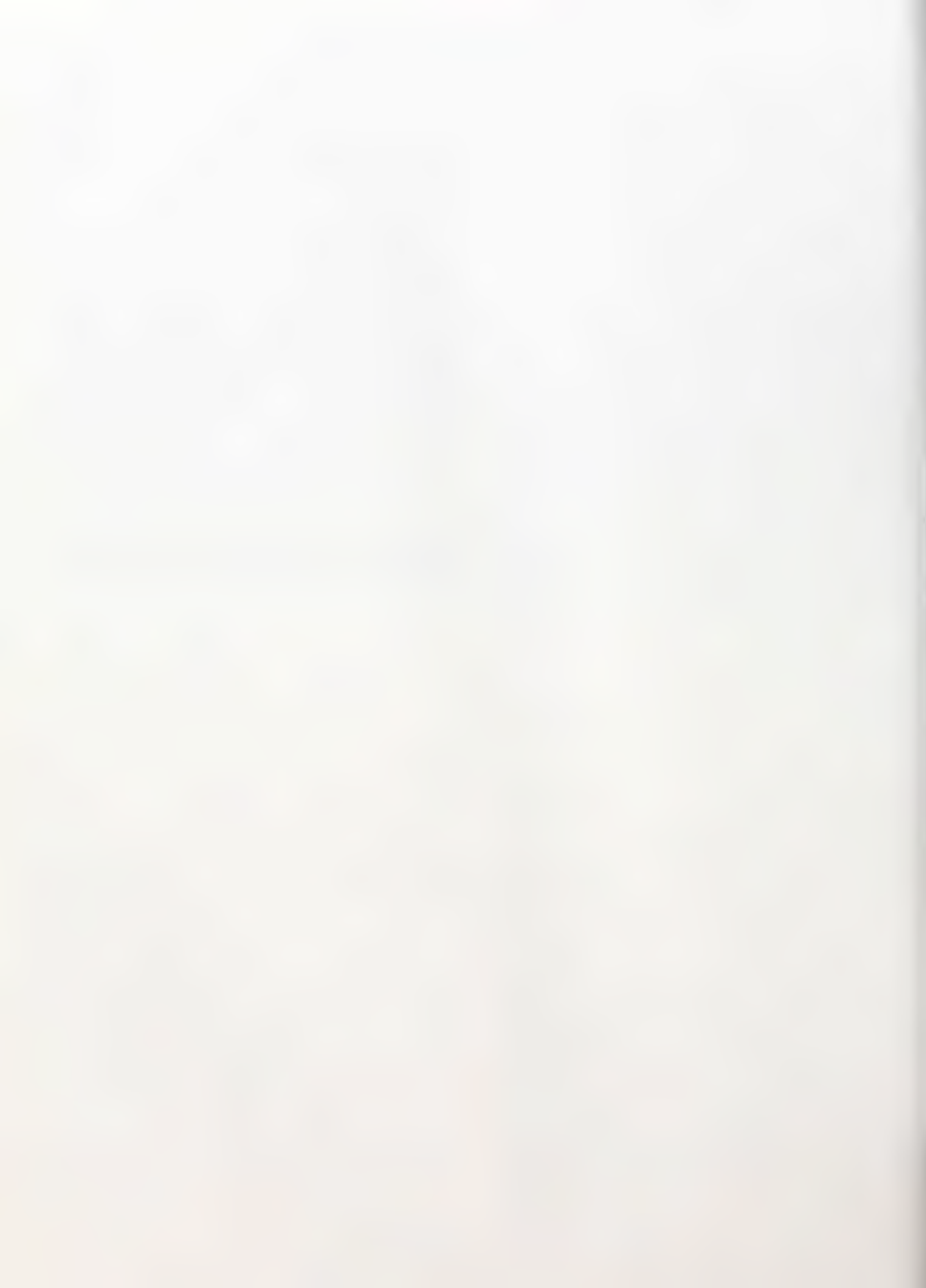
Model predictions of nitrogen load to the recharge areas (including those from the other Waquoit watersheds) fit measured data less well than those for the entire estuaries ($r^2 = 0.80$ vs. $r^2 = 0.96$) (Fig. 1, bottom right). Surprisingly, the best fit line in this case lies closer to the 1:1 line. The most likely explanation for the differences between the field and model-loading values is that the model is not spatially explicit and, therefore, cannot capture the effect of specific locations of land use within recharge areas. For example, two of the recharge areas from the Eel Pond watershed have the most divergent model predictions

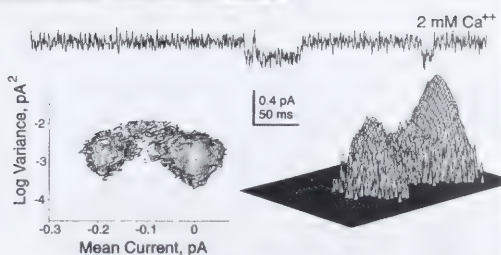
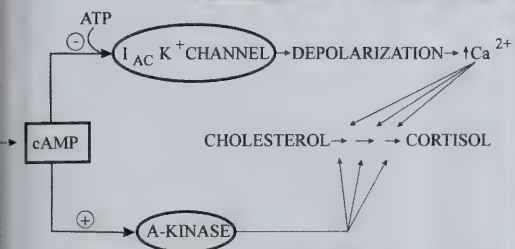
relative to field measurements. These two recharge areas are ones in which residential land use is concentrated near shore, which may increase actual relative to predicted nitrogen loads. Location effect may be less important as parcel size increases, and more patches of different land uses occur in different places on the catchment basin. The WBNLM seems most appropriate for larger areas—even whole watersheds—if we are concerned with scatter of the data, and for smaller parcels if we are concerned with predicting lines. At both spatial scales, WBNLM promises to be a useful tool for comparing watersheds, predicting effects of large-scale changes in land use, and evaluating nitrogen load to adjoining aquatic ecosystems.

Special thanks to Maggie Geist of the Waquoit Bay National Estuarine Research Reserve for working with us on the model, and to John Brawley for supplying data on land use. This work was supported by an NSF Research Experiences for Undergraduates grant.

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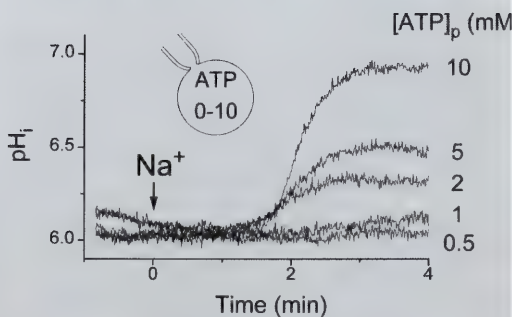
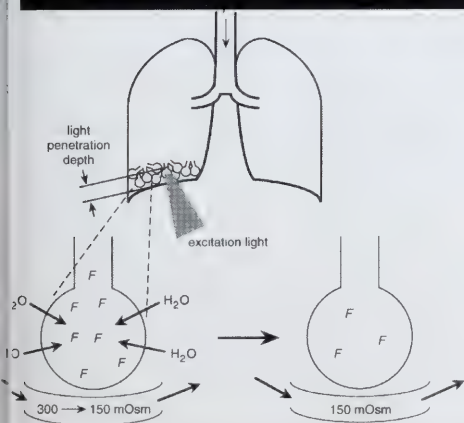
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A complete claw has regenerated from the eye socket of the crab *Cancer jordani*. The eyestalk was removed and tissues from the claw digits (dactyl and pollex) of the host crab were implanted into the socket. The results indicate that distal portions of the claw can regenerate a complete claw in a heterotopic site. See Kao and Chang, this issue, for further details.

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Are Echinoderm Egg Size Distributions Bimodal?

MARY A. SEWELL* AND CRAIG M. YOUNG

*Department of Larval Ecology, Harbor Branch Oceanographic Institution,
5600 U.S. 1 North, Fort Pierce, Florida 34946*

Marine invertebrates can be categorized into species that reproduce by producing either large numbers of small, energetically inexpensive eggs that become planktotrophic larvae, or fewer, larger eggs with more yolk and lecithotrophic development (1). The selective advantages of these alternative strategies were considered in a series of simple mathematical models by Vance (2, 3). These models predicted that intermediate egg sizes should have lower reproductive efficiency, and that only extreme egg sizes should be evolutionarily stable (2, 3). Specifically, Vance's models (2, 3), and later modifications (4–7), predict that eggs of marine invertebrates should have bimodal size distributions, reflecting the contrast between small egg/feeding and large egg/nonfeeding modes of development and the selection against intermediate egg sizes. Evidence for bimodality in egg size distributions is, however, equivocal, with unimodal distributions seen in the majority of comparative studies that are appropriate tests of the hypothesis (8–13). Bimodal distributions have been described only in a few groups of molluscs (4) and in asteroid and echinoid echinoderms (14). Here we test the prediction of bimodality in the holothuroid and ophiuroid echinoderms and show that although the natural log-transformed egg size distributions are visually unimodal, the holothurian egg size distribution is statistically composed of two discrete modes. Moreover, reexamination of the asteroid and echinoid egg size distributions (14) with the addition of data from more recent literature confirms that there are two statistical modes in the egg size distributions of these classes. Thus, in the phylum Echinodermata, there is a bimodal egg size distribution in three of the four classes in which this prediction can be tested.

In marine invertebrates there is a tradeoff between fecundity and the amount of energy that can be invested in each egg. Thus, as a rule, a species produces either many, small eggs with planktotrophic development or fewer, larger eggs with lecithotrophic development (1). As the size of eggs may differ between closely related species, egg size is assumed to be subject to considerable selective pressure (15–17). This has prompted interest in documenting patterns of egg size among species and higher taxa, and in exploring how factors such as phylogeny (15), fertilization biology (16, 18, 19), length of the pre-hatching (14, 20) and prefeeding periods (17), and size and organic content at metamorphosis (e.g., 14, 21) might be important for selection of egg size.

In early mathematical modeling Vance (2, 3) viewed planktotrophy and lecithotrophy as extreme forms of larval development, and his models suggested that only the extreme egg sizes would confer maximum reproductive efficiency (number of newly metamorphosed adults per unit of energy devoted to reproduction). Subsequently, the prediction of bimodality in egg sizes within taxa of marine invertebrates was tested by a number of authors using previously published or new data sets. Most of these studies showed unimodal egg size distributions (see also fig. 2 in ref. 22). Examples include prosobranch gastropods (9, 10), opisthobranchs (11), muricid gastropods without nurse eggs (12), Indo-Pacific *Conus* (13), bivalves (8), chitons (23), thoracican barnacles (24), and stomatopods (25). Bimodal distributions in egg size were described in American species of the genus *Crepidula* (4), in prosobranch gastropods from Danish waters (4), and in echinoid and asteroid echinoderms (14).

Many of the early studies used data sets that were not adequate for a valid test of the distribution patterns of egg size. We defined three criteria for appropriate data sets: (a) the data should include both planktotrophic (feeding) and lecithotrophic (nonfeeding) forms of develop-

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ment; (b) the egg sizes should be from species that span a broad range of habitats and a wide geographic range; and (c) the data should exclude species that provide additional maternal investment (e.g., nurse eggs, capsules). Criterion (b) is necessary because egg size may be correlated with habitat—for example, more planktotrophs in the tropics (1)—and use of species from a limited geographic area might under- or overemphasize a particular developmental type. Criterion (c) is necessary because Vance's models (2, 3) assume that reproductive energy is invested solely in the egg. Additionally, because the models (2, 3) were developed in terms of energetic content rather than egg size *per se*, for all data sets it is assumed that there is a correlation between egg size and energetic content.

The primary criterion, that planktotrophic and lecithotrophic forms be present, is violated by three of the taxa: thoracican barnacles (24) and stomatopods (25), neither of which groups include species with lecithotrophic development, and chitons (23), which do not include species with planktotrophic development. Criterion (b) may be violated by Ockelmann's bivalve data set (8) because planktotrophs are rare in East Greenland (26), in compilations of egg sizes in American species of *Crepidula* (4) and Danish prosobranchs (4), and in studies with low sample sizes (4, 23, 24, 25). Violation of criterion (c) has been avoided by removal of species using nurse eggs (12, fig. 2 in ref. 22). With the removal of studies that clearly violate criterion (a) there are unimodal egg size distributions in six taxa (8–13) and bimodal distributions in four taxa (4, 14).

The best known, and most widely cited, example of the bimodality in egg sizes within a taxon is the extensive data set compiled by Emlet *et al.* (14) for asteroid and echinoid echinoderms. This data set meets criteria (a)–(c) above, incorporating egg sizes from 89 asteroid species and 109 echinoid species from a variety of habitats. Moreover, in this phylum there is a strong correlation between egg size and energetic content (27). Of the four remaining classes of echinoderms, our criteria are not met in the crinoids, where there are no known planktotrophic species (28), or in the concentricycloids, where there are only two described species (29). In this paper we test the prediction of bimodality in egg sizes in the two remaining classes, the holothuroids and ophiuroids, which meet the criteria defined above, and we incorporate new data into the published egg size distributions of echinoids and asteroids (14).

The egg size data sets used in this analysis were compiled from the published literature and our own unpublished data, with the implicit assumption that all echinoderm eggs are basically alike. As noted by Turner and Lawrence (30), although egg diameter is an easy reproductive character to measure, there are shortcomings in

its use for theoretical discussions of life-history patterns. Firstly, many species have oblatel or prolately spheroidal eggs, so that eggs with the same diameter may have different egg volumes (30). We did not attempt to convert published egg diameters to volumes because in most cases only one of the two linear dimensions required for such a calculation was presented. Secondly, egg measurements made from histological sections will underestimate egg diameter because of tissue shrinkage during processing and the hydration of the egg during spawning (30). We used the diameters of both intraovarian and freshly spawned eggs in this paper to increase the sample sizes for statistical analysis ($n > 100$ species for all classes). Because the size classes in the raw (100 μm) and $\ln$ -transformed analyses ($\ln 0.25$) are relatively large, we assumed that intraovarian egg diameters would have a low probability of moving into an adjacent size class should a freshly spawned egg diameter be available. Thirdly, eggs differ in their organic components—lipid, protein, etc.—(27, 30) and our assumption that egg diameter is an effective measure of maternal investment may prove to be untenable.

Previous studies of egg size distributions in marine invertebrates have determined the number of modes in the distribution by visual appraisal. In this paper we instead made use of statistical techniques designed to differentiate length-frequency classes of fish populations (31). Histograms of egg diameter frequencies were generated for each class using the raw and $\ln$ -transformed diameters as shown in Figures 1–4. A modal analysis using the method of Schnute and Fournier (31) was then used to identify the Gaussian modes presented in these figures. Because the analysis is relatively insensitive to the dimensions of the size classes (31; B. Smith, pers. comm.), this method provides an objective way to examine the egg size distribution in a particular taxon and directly test the hypothesis of bimodality of egg sizes in marine invertebrates (2, 3). The complete raw data sets used for these analyses are available to interested readers from either of the authors.

Holothuroids show an extreme range in egg diameter, from a minimum of 50 μm in the apodid *Synaptula reciprocans* to a maximum of 4400 μm in the elasipod *Psychropotes longicauda* (Fig. 1A). About 80% of the species have eggs smaller than 1000 μm in diameter (Fig. 1A), with four modes in the size-frequency distribution (Table 1). The first mode is at an egg diameter of 194 μm (Fig. 1A, Table 1), and comprises species with eggs from 50 to about 300 μm with planktotrophic or unknown development (Fig. 1A). A second, but overlapping, mode is seen at 421 μm , with species with lecithotrophic, brooded, or unknown development (lecithotrophs: 150–950 μm ; Fig. 1A). A large number of species in the unknown category in this mode are dendrochirote holothurians with

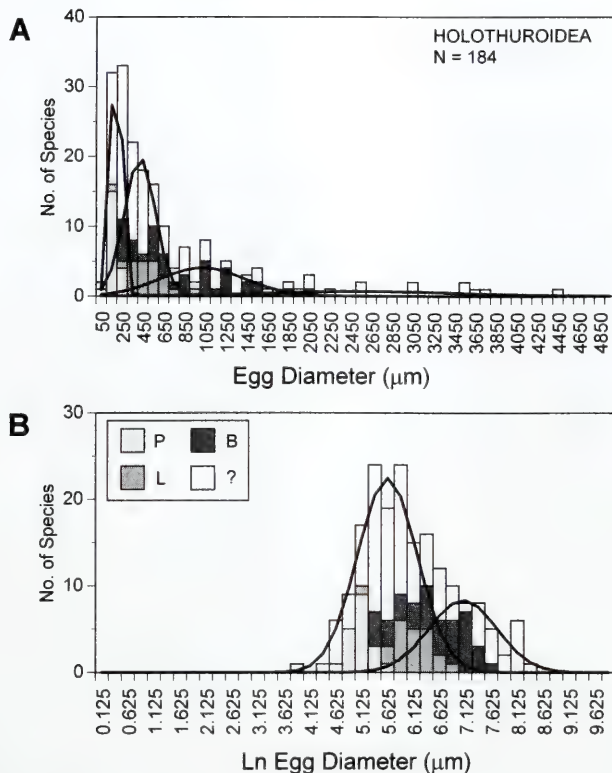


Figure 1. Egg size distribution pattern in the class Holothuroidea ($n = 184$). Information on the egg size of species of holothurians was compiled from the published literature and our own unpublished data. If more than one egg size was reported for a particular species, the maximum value was used in the figure. The type of larval development (planktotrophic, lecithotrophic, brooding) was based on the known development of that species from laboratory or field collections. This resulted in a large number of species with unknown developmental type; egg size was not used to infer developmental mode. Species were categorized as having an auricularia larva (planktotrophic), a doliolaria larva only (lecithotrophic), or internal/external brooding. Two species, *Holothuria floridana* and *Thyone fusus*, that are reported to have pelagic direct development were included with the lecithotrophs. The complete raw data set for this figure can be obtained from either of the authors. (A) Histogram of the untransformed egg diameters in the class Holothuroidea; x-axis shows the midpoint of the 100- μm size class. (B) Histogram of the natural log-transformed egg diameters in the class Holothuroidea; x-axis shows the midpoint of the $\ln 0.25$ size class. Part B follows Emlet *et al.* (14) in using the natural log transformation of egg diameter to combine species with large lecithotrophic eggs into a smaller number of categories. Legend to developmental type is as shown in part B: P = planktotrophic ($n = 20$); L = lecithotrophic ($n = 22$); B = brooded ($n = 38$); ? = unknown development ($n = 104$). Solid line differentiates modes identified in the statistical analysis (see Table I for details).

egg sizes in the 200–800 μm range. As there are no known planktotrophs in this order (32), this mode may additionally include 20 lecithotrophic species. The last two modes, at 1024 and 2603 μm , are primarily composed of species that are lecithotrophic, brood, or have unknown

development type (Fig. 1A)—the majority of the latter species are elapsid holothurians and may have direct development (33). Thus, in the holothurians the first mode contains mainly species with planktotrophic development; the following three modes are species with nonfeeding

Table 1

Results of modal analyses for egg size distributions in asteroid ($n = 149$), echinoid ($n = 181$), holothuroid ($n = 184$), and ophiuroid composites ($n = 182$)

Class	No. of modes	Mean egg diameter (μm)	SD	No. of species in mode	K-S test statistic
(a) Raw numbers					
Asteroidea	4	162	29	55.32	0.026
		475	89	25.99	
		1008	206	55.42	
		2472	806	12.25	
Echinoidea	4	102	15	91.99	0.014
		317	140	20.76	
		1157	160	14.00	
		2000	18	4.00	
Holothuroidea	4	194	45	51.09	0.021
		421	147	74.40	
		1024	409	41.42	
		2603	970	16.50	
Ophiuroidea	3	165	50	58.46	0.055
		450	210	71.34	
		1528	3	1.00	
		(b) Natural log transformation			
Asteroidea	2	5.03	0.24	55.32	0.040
		6.75	0.58	93.68	
Echinoidea	2	4.60	0.25	89.07	0.043
		6.22	0.92	41.93	
Holothuroidea	2	5.62	0.57	129.43	0.018
		7.08	0.65	54.57	
Ophiuroidea	1	5.46	0.69	132	0.051

The number and position of modes within each distribution were determined using a computer program developed by Dr. B. Smith (Environment Canada, Vancouver) using the method of Schnute and Fournier (31). For these analyses the mean and standard deviation of the modes were assumed to be independent of each other, and were not constrained to conform to any particular function (31). Competing models were ranked using the χ^2 procedure suggested by Schnute and Fournier (31), in combination with likelihood ratio tests (39) and the Akaike Information Criterion (AIC, 40). The table presents the parameters of the modal structures that best explained the observed data: the number of statistical modes in each distribution, the mean egg diameter and standard deviation of each mode, and the number of species in mode (the proportion of observations in each mode multiplied by the total number of species in the distribution). The Kolmogorov-Smirnov (K-S) test statistic (41) was used as a measure of the fit of the predicted model to the observed distribution, and is not rejected at the 0.05 level in any case.

(planktonic or brooded lecithothrophs) or unknown development.

The natural log-transformed distribution appears visually to be unimodal with a slight right skew (Fig. 1B). Statistical analysis, however, reveals two modes with means of $\ln 5.62$ and 7.08 respectively (Table 1). There is no clear demarcation between planktotrophic, lecithothrophic, and brooding species, with representatives of all

three developmental types in the first mode (Fig. 1B). The second mode, however, is almost exclusively composed of species with lecithothrophic or brooded development.

Examination of the egg size distribution in the ophiuroids reveals a similar distribution in the untransformed egg sizes (Fig. 2A). Ophiuroid eggs are smaller than holothuroid eggs, ranging in diameter from $50 \mu\text{m}$ in *Ophiactis kroeyeri* to $1500 \mu\text{m}$ in *Astrospartus mediterraneus* (Fig. 2A). Ninety-five percent of the species produce eggs smaller than $800 \mu\text{m}$, and only two species have egg sizes greater than $1000 \mu\text{m}$ (Fig. 2A). There are three statistical modes in the egg size distribution: the first, at $165 \mu\text{m}$, of species with primarily planktotrophic (80 – $185 \mu\text{m}$) development; the second, at $450 \mu\text{m}$, of species with abbreviated (150 – $350 \mu\text{m}$), brooded (100 – $1000 \mu\text{m}$), or unknown development; and the third, at $1528 \mu\text{m}$, which results from a single species (*Astrospartus mediterraneus*) with unknown development (Fig. 2A, Table 1).

The $\ln$ -transformed egg size distribution in the ophiuroids appears visually to be unimodal (Fig. 2B) and similar in shape to that observed in the holothuroids (Fig. 1B). Although there are lower numbers of species with eggs in the $\ln 4.75$ – 5.0 size class (115 – $148 \mu\text{m}$), there is only one statistical mode ($\ln 5.46$, Table 1). In this class, therefore, all developmental types are included within the single mode of the distribution.

In literature published since the review of Emlet *et al.* (14) and in our own unpublished data, we added egg sizes for 61 asteroid species. Asteroids have four modes in the untransformed egg size distributions (Fig. 3A, Table 1). The first mode is of species with primarily planktotrophic development; the following three modes comprise species with lecithothrophic, brooded, or unknown development (Fig. 3A).

The addition of the new asteroid data to the natural log-transformed egg size distribution does not alter the bimodality described by Emlet *et al.* (14). Statistically there are two separate modes, at $\ln 5.03$ and 6.75 (Fig. 3B, Table 1)—the first (Fig. 3B) contains almost exclusively planktotrophic species (79%), with one lecithothrophic species (*Astropecten latespinosus*) and three brooders, two of which are intraovarian brooders, *Patriella vivipara* and *P. parvivipara* (34). The second mode (Fig. 3B) is predominantly lecithothrophic (62%) and brooding (12%) species, with the exception of the planktotrophic *Porania antarctica* (35). The asteroid species with the exceptional developmental type for that mode are often species with rare developmental types. For example, *Astropecten latespinosus* ($300 \mu\text{m}$) has an abbreviated lecithothrophic development, lacks both a bipinnaria and a brachiolaria stage, and is in the plankton for only about 4 days (36). *Porania antarctica* similarly has a large egg ($550 \mu\text{m}$) and

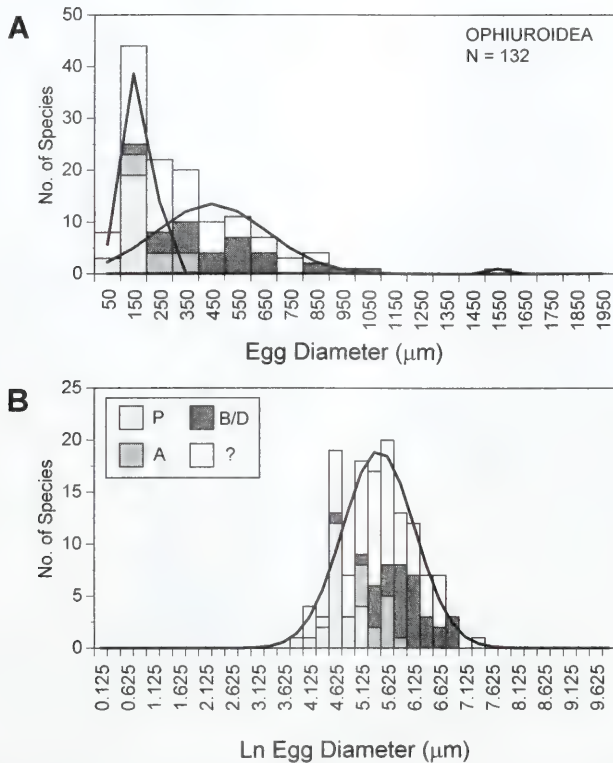


Figure 2. Egg size distribution pattern in the class Ophiuroidea ($n = 132$). Information on the egg size of species of ophiuroids was compiled from Hendler (109 ophiuroid species, ref. 38) and later published literature. The type of larval development was based on Hendler's classification (ref. 38, p. 456): planktotrophic = with a relatively long-lived ophiopluteus larvae; abbreviated = reduced ophioplutei, embryos in an attached fertilization envelope, lecithotrophic vitellaria larvae, or demersal larvae; brooded/direct = viviparous, internal or external brooding, or with an egg capsule. The complete raw data set for this figure can be obtained from either of the authors. (A) Histogram of the untransformed egg diameters in the class Ophiuroidea; x-axis shows the midpoint of the 100- μm size class. (B) Histogram of the natural log-transformed egg diameters in the class Ophiuroidea; x-axis shows the midpoint of the ln 0.25 size class. Legend to developmental type is as shown in part B: P = planktotrophic ($n = 22$); A = abbreviated ($n = 12$); B/D = brooded/direct ($n = 31$); ? = unknown development ($n = 67$). Solid line differentiates modes identified in the statistical analysis (see Table I for details).

planktotrophic development, but has the shortest period of development (65 days) of any of the Antarctic asteroids studied by Bosch and Pearse (35). For these species, particularly, it would be of interest to examine whether the energetic content or biochemical composition of their eggs differs from those of other asteroids of the same developmental type.

In the four echinoderm classes analyzed, it is only the

asteroids that show two separate, distinct, and statistically differentiated modes (Fig. 3B). There are few species with egg sizes in the size class ln 5.5 to 5.75, equivalent to egg diameters of 245 to 314 μm (Fig. 3B).

Twenty-two egg sizes were added to the echinoid data set of Emlet *et al.* (14). The untransformed distribution comprises four modes (Fig. 4A, Table I). The first two modes, at 102 and 317 μm , overlap considerably and are

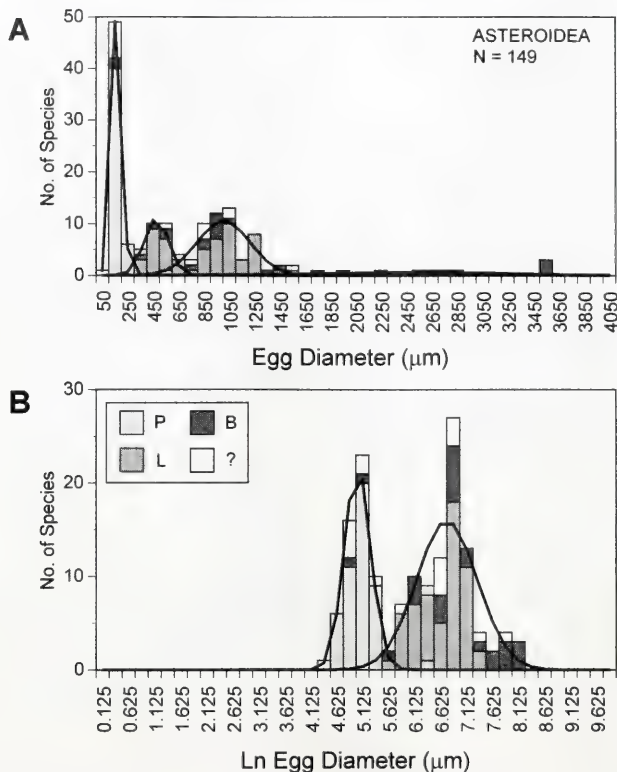


Figure 3. Egg size distribution pattern in the class Asteroidea ($n = 149$). Information on the egg size of species of asteroids derived from Emlet *et al.* (14) and more recent literature. The complete raw data set for this figure can be obtained from either of the authors. (A) Histogram of the untransformed egg diameters in the class Asteroidea; x-axis shows the midpoint of the 100- μm size class. (B) Histogram of the natural log-transformed egg diameters in the class Asteroidea; x-axis shows the midpoint of the $\ln 0.25$ size class. Legend to developmental type is as shown in part B: P = planktotrophic ($n = 47$); L = lecithotrophic ($n = 57$); B = brooded ($n = 26$); ? = unknown development ($n = 19$). Solid line differentiates modes identified in the statistical analysis (see Table I for details).

composed of planktotrophic, facultative planktotrophic, and some lecithotrophic species (Fig. 4A). The last two modes are of lecithotrophic and brooding species (Fig. 4A).

The $\ln$ -transformed histogram shows statistically two modes (Fig. 4B, Table I). The first ($\ln 4.60$) comprises 68% of the species and is composed almost entirely of planktotrophic species, with the facultative planktotroph *Encope michelini* (37) at the point of overlap between modes 1 and 2 (Fig. 4B). The second mode, at $\ln 6.22$, has a high standard deviation (Table I) and includes two species of facultative planktotrophs (*Clypeaster rosaceus*,

Brisaster latifrons, 14) as well as all the lecithotrophic and brooding species (Fig. 4B).

Two conclusions can be drawn from the statistical analyses presented here. Firstly, in all classes the untransformed egg size distributions show three to four modes, the first being primarily of planktotrophic species, and the remaining two to three containing mainly species with nonfeeding development (*i.e.*, lecithotrophic, abbreviated, brooded, or direct development). Secondly, the $\ln$ -transformed distributions are bimodal in the holothurians, asteroids, and echinoids, but unimodal in the ophiuroids. In

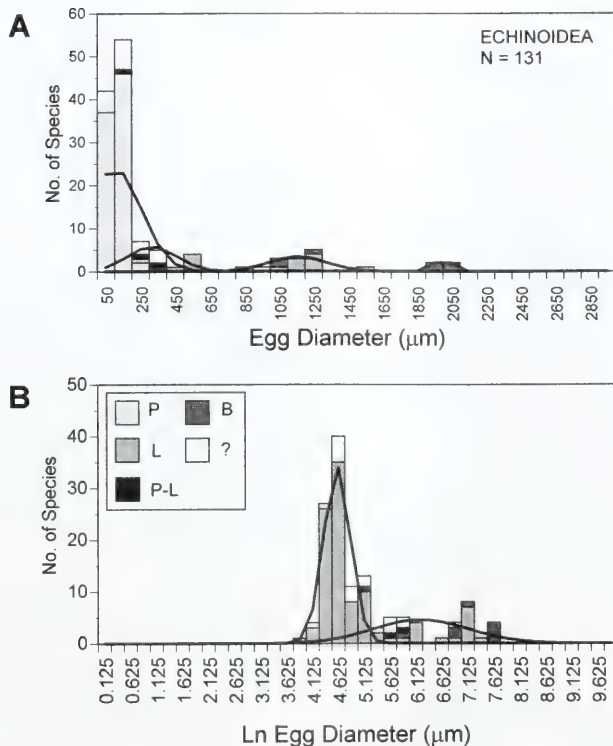


Figure 4. Egg size distribution pattern in the class Echinoidea ($n = 131$). Information on the egg size of species of echinoids derived from Emlet *et al.* (14) and more recent literature. The complete raw data set for this figure can be obtained from either of the authors. (A) Histogram of the untransformed egg diameters in the class Echinoidea; x-axis shows the midpoint of the 100-µm size class. (B) Histogram of the natural log-transformed egg diameters in the class Echinoidea; x-axis shows the midpoint of the ln 0.25 size class. Legend to developmental type is as shown in part B: P = planktotrophic ($n = 85$); L = lecithotrophic ($n = 16$); P-L = facultative planktotroph ($n = 3$); B = brooded ($n = 8$); ? = unknown development ($n = 19$). Solid line differentiates modes identified in the statistical analysis (see Table 1 for details).

those classes with a bimodal distribution, it is only the asteroids and echinoids that show a demarcation between modes with feeding and nonfeeding development. In the holothuroids, the modes are less visually distinct, and lecithotrophic and brooding developmental types are found in both modes.

The egg size data examined here (Figs. 1–4) illustrate the need to view marine invertebrate development as a reproductive continuum (5, 17, 22). Histograms of the egg size distribution show species with planktotrophic development forming a distinctive group at smaller egg sizes (max. egg size <300 µm; Figs. 1–4). However, in

all classes, there is some overlap in egg size between these planktotrophic species and species with lecithotrophic or brooded development (Figs. 1–4). Egg sizes of species with “intermediate” forms of development, such as facultative planktotrophy (*e.g.*, 17) or modified benthic development (*e.g.*, *Patiriella exigua*, egg 400 µm; 34), generally fit within the mode for other lecithotrophic and brooding species (Figs. 3 and 4).

An important direction for future research on echinoderm eggs is examination of the assumption made here, and in other comparative studies (*e.g.*, 14), that all eggs are basically equal (27). This question should be ad-

dressed at two levels. Firstly, within an echinoderm class we need information on the energetic content and biochemical composition of species with a particular developmental type. For example, in the Holothuroidea it would be of interest to determine whether the eggs of known lecithotrophs (egg diameter 150–950 μm) differ in any way from those of the planktotrophic species or from the large (150–4400 μm) eggs of the deep-sea elisipods that are suspected of having direct development (33). Secondly, when sufficient data have been compiled, it would be of interest to compare egg characters (size, energetic content, and composition) between echinoderm classes. In a recent review of the relationship between egg size and energetic content in echinoderms, Jaekle (27) showed that there were average differences in egg size, energetic content, and biochemical composition between developmental types, but the low number of available studies made it necessary to combine egg parameters from different echinoderm classes. With increasing study on the energy content and biochemical composition of eggs of the less well known echinoderm classes (holothuroids, ophiuroids, crinoids) we may be better able to interpret patterns of egg size distribution (Figs. 1, 2) and formulate or test new hypotheses on factors important for the selection of egg size.

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Spermiogenesis and Sperm Structure in Relation to Early Events of Fertilization in the Limpet *Tectura testudinalis* (Müller, 1776)

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Abstract. Spermiogenesis and fertilization in the limpet *Tectura testudinalis* (Mollusca: Archaeogastropoda: Patelloidea) were examined using scanning and transmission electron microscopy, as well as light microscopy. Spermiogenesis was similar to that described for other Lottiidae (Patelloidea), but some key differences were noted.

We observed that the acrosomal vesicle forms on the plasma membrane from several Golgi-derived proacrosomal vesicles during the early spermatid. Evidence from anti-actin antibody staining of a related species, *T. scutum*, indicates that the acrosome probably contains G-actin in three different locations, but there are no preformed actin filaments. The flagellum of mature sperm tapers to a thin, filamentous end-piece.

The mature egg of *T. testudinalis* is enclosed by a thin vitelline layer and a thick jelly coat bound by follicle cells. Where a mature sperm encounters the intact jelly coat, its acrosome tip may elongate, up to twice its original length. The acrosome reaction is not induced, but by this mechanism the acrosome bridges the jelly coat and extends down far enough to contact the vitelline layer. During the elongation of the acrosome, microfilaments are formed inside the tip. As well, new membrane required for this process is supplied by elimination of any slack in the plasma membrane and also perhaps by spontaneous formation of vesicles inside the acrosome. Once the tip of the sperm contacts and binds with the vitelline layer it undergoes the acrosome reaction, in which the plasma and acrosomal membranes fuse and roll back in a manner typical of scaphopods and some polychaetes. Sperm also may bind directly with the vitelline layer when

the jelly coat is absent. Furthermore, the acrosome tip may undergo spontaneous fusion with a naked egg microvillus that formerly connected with a follicle cell process. Thus, the sperm either individually or collectively are equipped to deal with any one of several potential routes to successful fertilization of the egg.

Current theories of gastropod phylogeny place the limpets as basal to the Gastropoda, but new evidence presented here supports the idea that they are a divergent group, with some unique innovations in sperm design.

Introduction

The process of fertilization in invertebrates typically involves intricate structural responses of both sperm and egg; these are often unique to a particular species and function to regulate sperm-egg recognition as well as sperm entry into the egg (see reviews by Dan, 1967; Colwin and Colwin, 1967; Epel, 1978; Monroy and Rosati, 1983; Tilney, 1985; Koch and Lambert, 1990; Buckland-Nicks, 1995). These mechanisms, often interdependent, work in conjunction to facilitate sperm penetration of the outer protective envelopes of the egg prior to a successful fertilization (Tilney, 1985).

In free-spawning marine invertebrates, ripe unfertilized eggs have one or more extracellular envelopes. In some groups these egg envelopes play a key role in the induction of the sperm acrosome reaction, sperm-egg binding, and the exclusion of supernumerary sperm (see reviews by Colwin and Colwin, 1967; Epel and Vacquier, 1978; Monroy and Rosati, 1983; Tilney, 1985; Longo, 1987; Sato and Osanai, 1990). Just as the egg plays a role in controlling sperm penetration, the sperm itself undergoes a complex series of chemical (exocytotic) and morphological changes that permit penetration of the egg envelopes

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and fusion with the egg membrane. Classic studies on echinoderms have shown that contact with the egg jelly coat induces exocytosis of the acrosomal vesicle, which releases lysins that dissolve the egg envelopes (see reviews by Colwin *et al.*, 1975; Tilney, 1985; Epel and Vacquier, 1978). Simultaneous with the exocytosis of lysins is the rapid extension of an acrosomal process that projects the inner acrosomal membrane into contact with the vitelline layer overlying the egg microvilli. Typically this independent process involves either the rapid polymerization of globular to filamentous actin to form a rod-like structure (Schroeder and Christen, 1982; Tilney, 1985), or the inter-sliding of actin filaments in a pre-formed rod, causing its extension, as in bivalves (Tilney, 1985; Tilney *et al.*, 1987) and some archaeogastropods (Lewis *et al.*, 1980; Shiroya *et al.*, 1986; Shiroya and Sakai, 1993). Species-specific recognition and binding with the vitelline layer and its subsequent dissolution by other acrosomal lysins results in fusion when specific receptors on the inner acrosomal membrane contact their counterparts on the egg membrane.

The exocytosis of acrosomal lysins and the extension of an acrosomal process (or its equivalent as in chitons [Buckland-Nicks *et al.*, 1988]) are typical of mechanisms used to penetrate the egg envelopes of free-spawning marine invertebrates; however, they may not always be necessary even when present (see discussion in Monroy and Rosati, 1983). In some species with reduced or absent jelly coats, the evagination of the inner acrosomal membrane may not require an acrosomal process, or if so, a very reduced one (Dufresne-Dubé *et al.*, 1983; Sato and Osanai, 1983, 1990).

Among molluscs, studies of early events of fertilization have largely been restricted to species with preformed axial rods, such as bivalves (Nijima and Dan, 1965; Hylander and Summers, 1977; Tumboh-Oeri and Koide, 1982; Longo, 1973, 1987), and some archaeogastropods (Lewis *et al.*, 1980; Sakai *et al.*, 1982; Shiroya and Sakai, 1984, 1992; Shiroya *et al.*, 1986, 1989). In other gastropod molluscs, these early events are virtually uncharted. This is partly because many gastropods fertilize internally and the conditions, which involve capacitation of the sperm as well as specific changes to the egg, are difficult to simulate in the laboratory. Thus, external fertilization was an important consideration in our selection of the limpet *Tectura testudinalis* (Gastropoda: Patelloidea: Lotiidae) as a suitable model in which to study early events of fertilization.

The Patelloidea are of particular interest in light of the ongoing debate as to their phylogenetic status among 'Archaeogastropoda' *sensu lato* (Branch, 1985; Haszprunar, 1988; Hodgson, 1995; Lindberg, 1988; Buckland-Nicks and Scheltema, 1995; Hodgson *et al.*, 1996). Sperm structure has recently been found to provide a series of

characters useful for testing extant hypotheses on phylogenetic relationships (Healy, 1988, 1996; Giusti *et al.*, 1990; Justine, 1991; Jamieson *et al.*, 1991; Jamieson, 1991) and is one of the main reasons for concluding that in several animal groups external fertilization with 'primitive' sperm is not necessarily primitive (Jamieson, 1991; Rouse and Fitzhugh, 1994; Buckland-Nicks, 1994). Rather, internal fertilization with introsperm may be basal to the Bilateria (Buckland-Nicks and Scheltema, 1995). Justine (1991) emphasized the importance of studying early events of spermiogenesis to obtain a complete understanding of how sperm organelles are formed. There have been many studies of mature sperm ultrastructure in Patelloidea (Azevedo, 1981; Kohnert and Storch, 1983; Koike, 1985; Smaldon and Duffus, 1985; Hodgson and Bernard, 1988, 1989; Jamieson *et al.*, 1991; Hodgson and Chia, 1993; Sousa and Oliveira, 1994; Hodgson *et al.*, 1996), but only one detailed study of spermiogenesis (Hodgson and Bernard, 1988).

What has been overlooked so far is that a number of taxon-specific changes in sperm ultrastructure occur during the early events of fertilization. Thus, the study of fertilization, which is intrinsically interesting, can also provide new characters for improving the accuracy of current hypotheses on phylogenetic relationships. Our detailed description of spermiogenesis in *T. testudinalis* broadens the base of knowledge and provides new morphological characters for use in phylogenetic analysis of the Archaeogastropoda. Furthermore, it documents, for the first time in a limpet, the basic process of sperm-egg interaction—which turns out to be surprisingly unusual.

Materials and Methods

Spermiogenesis and mature sperm ultrastructure

Specimens of *Tectura testudinalis* (Müller, 1776) (see Lindberg, 1986) were collected from the intertidal regions at Port Bickerton, Nova Scotia, between June and September 1994. *T. testudinalis* is gonochoric (dioecious), and males and females were sorted on the basis of ventral foot coloration. In the male, a whitish band corresponding to the testis is visible on the left underside of the foot, which corresponds to the right (gonadal) side of the limpet. In the female, this same region is reddish. *T. scutum* (Rathke, 1833) was collected from the intertidal zone on San Juan Island, Washington, in August 1995 and was used only to study actin distribution in the acrosome (see Epifluorescence microscopy, this section).

Small portions of the male testis were prepared for transmission electron microscopy (TEM) by excising testis from the body mass, mincing it into pieces of about 1 mm³, fixing in ice-cold 2.5% glutaraldehyde (brought to pH 8.0 using NaOH), in Millipore-filtered seawater (MFSW), and allowing the tissue to come to room temper-

ature overnight. The primary fixative was removed, and specimens were washed in 0.2 M sodium cacodylate buffer (pH 7.6) for 10 min, then transferred to ice-cold 1.25% OsO₄ in 0.2 M sodium cacodylate buffer (marine rinse) as a secondary fixative for 1 h. Subsequent washing in 0.2 M sodium cacodylate buffer for 15 min was followed by dehydration in a graded ethanol series (30, 50, 70, 80, 90, 95, 100 × 3), for 10 min in each concentration. Pieces were then infiltrated in 1:1 Spurr's: 100% ethanol mixture and left overnight in a desiccator.

The following day, specimens were placed in 100% Spurr's resin for 6–8 h, transferred to dried polyethylene BEEM capsules containing fresh Spurr's medium, and baked overnight at 70°C. Polymerized blocks were removed from the capsules, rough trimmed, and semi-thin sectioned with a glass knife mounted in a Sorvall MT-2 ultramicrotome. Thick sections were stained with Richardson's stain. When an area of the egg was encountered with many juxtaposed sperm, suggesting polyspermy, the block was thin sectioned with a diamond knife (Diatome) mounted in a Sorvall MT-2C ultramicrotome. Sections with silver-gold interference color were picked up on naked 150-mesh copper grids, stained sequentially with saturated aqueous uranyl acetate for up to 30 min followed by lead citrate for 10 min, and examined in a Philips 300 transmission electron microscope.

Early events of fertilization

Preparation of gametes. Ripe animals from the field collections were "stripped" to obtain gametes. The entire gonad was cut away from the gut and body mass of both the males and the females and placed into separate clean petri dishes. The ovary was cut open and gently prodded with a blunt pointer while being flushed with 0.45- μ m MFSW to expel eggs. Eggs were transferred in clean MFSW to a small centrifuge tube and gently centrifuged to separate any tissue debris into the supernatant, which was removed and replaced with fresh MFSW.

The testis was macerated dry to expel some sperm concentrate as well as to reduce premature activation by preventing contact with seawater. Sperm concentrate could be stored in the refrigerator in sealed Eppendorf vials for 1 or 2 h or used immediately by gently drawing several drops into a Pasteur pipette and adding them to a vial containing 10 ml of MFSW to activate them. This activated sperm concentrate was used within 10 min for fertilization of eggs.

Fertilization. In an attempt to induce polyspermy for microscopic study, activated sperm concentrate was added to a portion of washed eggs in a small vial and mixed well by gentle agitation. Mixing by pipetting was avoided because it damages sperm acrosomes. Fertilized eggs were fixed and dehydrated as previously described

for gonads. Fertilization success, as determined by verifying that fertilized eggs underwent cleavage, was considerably improved by prior treatment of eggs with 5-hydroxytryptamine (5HT) (Juneja *et al.*, 1993) and only slightly improved by pretreatment with alkaline seawater (pH 9.0) (Lewis *et al.*, 1980). Eggs pretreated with 5HT also showed some improvement in apparent polyspermy when subjected to sperm concentrates. Batches of eggs that were treated with Hoechst 33342 DNA stain were examined with UV light in an Olympus BH2 light microscope equipped with epifluorescence. If many sperm nuclei appeared juxtaposed with the egg membrane, indicating polyspermy, the rest of that batch would be fixed for electron microscopy, as before, with the following modifications for scanning electron microscopy (SEM).

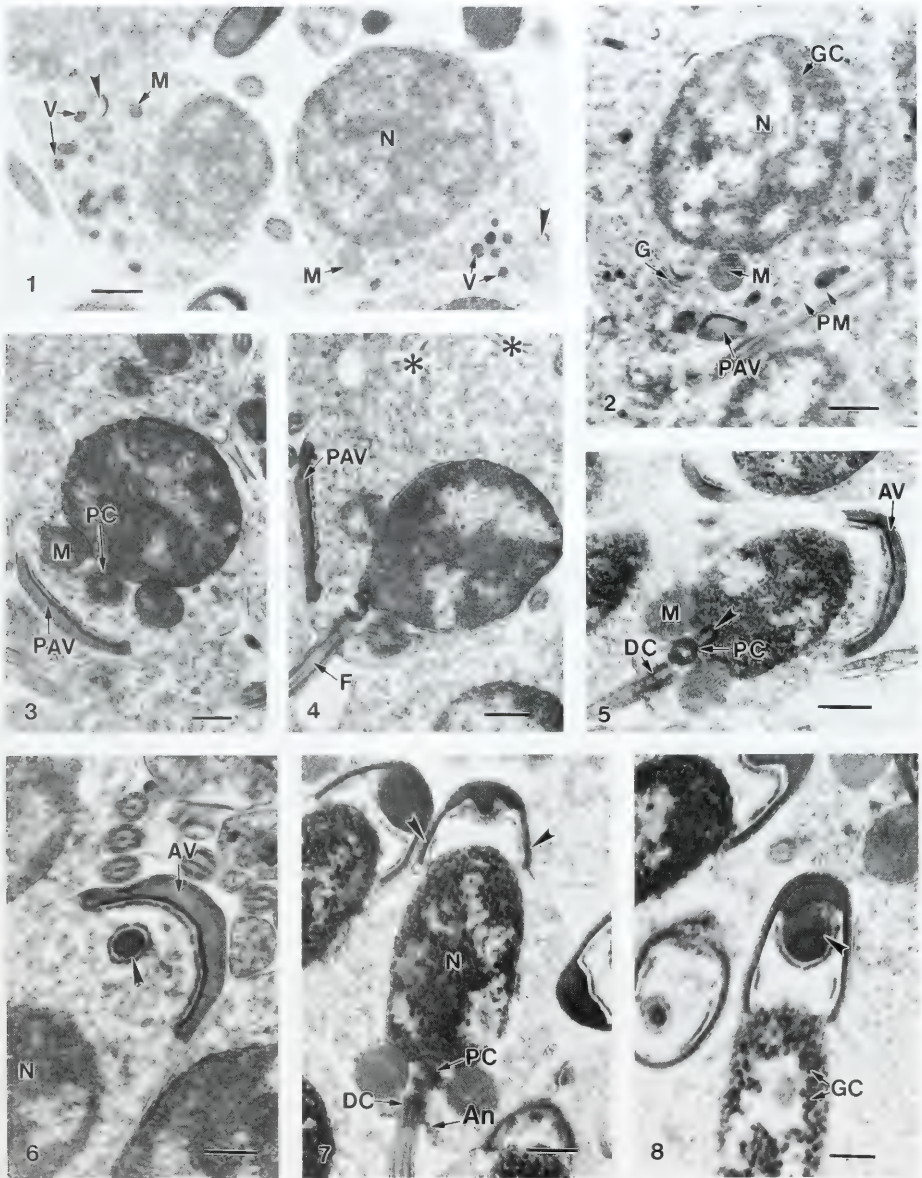
Scanning electron microscopy. Following dehydration, fertilized eggs were critical-point dried (Samdri) from 100% ethanol. The dried eggs were mounted on "JEOL" or "Cambridge" aluminum stubs with double-sided carbon sticky tape (Pelco). The stubs were coated with gold in a Polaron SC502 sputter coater and examined using a JEOL 5300 scanning electron microscope operated at 15 kV.

Epifluorescence microscopy. Samples of sperm of *T. scutum* were fixed for 30 min in 4% paraformaldehyde in 0.45- μ m MFSW and mounted on coverslips coated with poly-L-lysine (Sigma #A-4700). The coverslips were rinsed in PBS (phosphate buffered saline) three times and incubated for 1 h in anti-actin (primary) antibody developed in mouse (1/200 dilution) (Sigma). Coverslips were washed three times with PBS (15 min each wash) and incubated in rabbit anti-mouse IgG conjugated to FITC (1/200 dilution) (Sigma #F-7506) for 1 h. Coverslips were again washed three times in PBS and then mounted in PBS in glycerol with DABCO as an anti-bleaching agent. Controls were treated the same except that the primary antibody was omitted. Permanent slides were made by sealing stained coverslips to slides with "Wet and Dry" nail varnish. Prepared slides were examined in a Nikon Optiphot 2 fluorescence light microscope fitted with FITC filters. Micrographs were taken with TMax 400 film (Kodak) exposed at 800 ASA and developed for normal times in D76 developer (Kodak).

Results

Spermiogenesis

In early spermiogenesis, the Golgi body secretes numerous vesicles of varying density, some of which fuse to form a proacrosomal vesicle (Fig. 1), which becomes ovoid and attaches to the plasma membrane by some granular material (Fig. 2). Electron-dense material develops on the intracellular side of the proacrosomal vesicle as it enlarges and elongates (Figs. 2–4). While the Golgi



Figures 1-8. Transmission electron micrographs of spermiogenesis in *Tectura testudinalis*.

Figure 1. Early spermatid with large spherical nucleus (N), small mitochondria (M), and dense Golgi-derived vesicles (V) located at presumptive posterior of cell adjacent to small proacrosomal vesicle (arrow-heads). Scale bar = 0.5 μ m.

body remains basal, the maturing acrosomal vesicle migrates along the plasma membrane to the apex of the nucleus (Figs. 4, 5) and becomes cup-shaped with the concave side facing the nucleus (Figs. 5–8). Later in development, an invagination of the inner acrosomal membrane expands to form the posterior lobe (Fig. 8). This posterior lobe extends into the subacrosomal space (Fig. 9) and continues to elongate until it abuts some flocculent material on top of the nucleus (Fig. 10). Sometimes sections show a separate spherical vesicle in the subacrosomal space (Fig. 6), but this is interpreted as a cross section of the developing posterior lobe.

Early in spermiogenesis, many small mitochondria are distributed throughout the cell (Fig. 1). As the spermatid matures, the mitochondria aggregate posteriorly at the base of the nucleus (Figs. 5, 7), and eventually fuse to form a ring of four spherical mitochondria enclosing the proximal and distal centrioles (Figs. 5, 7, 11). The proximal centriole lies perpendicular to the apex of the distal centriole and becomes attached by fibrogranular material to a posterior invagination of the nucleus, termed the nuclear fossa (Figs. 5, 7). Extending posteriorly from the distal centriole is the elongating flagellum (Fig. 4).

The large spherical nucleus of the early spermatid comprises heterogeneous aggregations of granular chromatin (Figs. 1, 2). As the spermatid matures, the nucleus elongates in the anterior-posterior axis and the chromatin condenses to a coarse granular appearance (Figs. 7, 8). The cytoplasm of the developing spermatids in a cohort is joined by cytoplasmic bridges (Fig. 4), which are eliminated together with the residual cytoplasm when sperm are liberated into the lumen.

Ultrastructure of mature sperm

Mature sperm have an overall length of about 42 μm . The sperm are divided into four regions: head, mid-piece,

principal-piece, and end-piece. The head region comprises a conical acrosome measuring about $3.1 \mu\text{m} \times 0.7 \mu\text{m}$ and, posterior to this, a bullet-shaped nucleus of $2.6 \mu\text{m} \times 1.0 \mu\text{m}$, slightly tapered towards the apex (Figs. 10, 14). The total length of the head region is 5.7 μm . The mid-piece is 1.0 μm in length, comprising a ring of four mitochondria enclosing the centriolar complex, and includes the annulus in a "collar" encircling the flagellum (Fig. 14). The principal piece and end-piece have an overall length of 35.0 μm , of which the end-piece constitutes 5.2 μm .

The elongate acrosome cone exists as a two-part structure comprising an anterior portion, 1.4 μm in length, and a posterior portion containing the subacrosomal space, 1.2 μm in length (Fig. 10). The anterior acrosome is characterized by a layering of electron densities and granularities (Fig. 10). A central invagination of the inner acrosomal membrane extends posteriorly as a lobe into the subacrosomal space and abuts flocculent material at the anterior tip of the nucleus (Fig. 10 and inset). No microfilaments were visible within the lobe or the anterior acrosome. The subacrosomal space consists of a less electron-dense material that lacks the stratification of the anterior acrosome, but houses the invaginated posterior lobe.

The nucleus comprises homogeneously dense chromatin with some lacunae and lies in a small posterior fossa that houses the centrioles (Fig. 10). The mid-piece is located posterior to the nucleus and consists of a ring of four spherical mitochondria surrounding the centrioles (Figs. 11, 14). The proximal centriole is oriented perpendicular to the distal centriole, from which emanates the axial flagellum. A centriolar satellite complex comprised of nine spoke-like satellites connects the distal centriole with the annulus, which is attached to the plasma membrane, later forming a distinct collar posterior to the mid-piece (Fig. 14). The flagellum has a conventional 9 + 2

Figure 2. Early spermatid with aggregations of granular chromatin (GC) in nucleus (N). Some dense vesicles secreted from Golgi body (G) have fused posteriorly to form an ovoid proacrosomal vesicle (PAV), which is attached to plasma membrane (PM). Mitochondrion (M). Scale bar = 0.5 μm .

Figure 3. Spermatid with oblong proacrosomal vesicle (PAV) located posteriorly in cell. Mitochondrion (M), proximal centriole (PC). Scale bar = 0.5 μm .

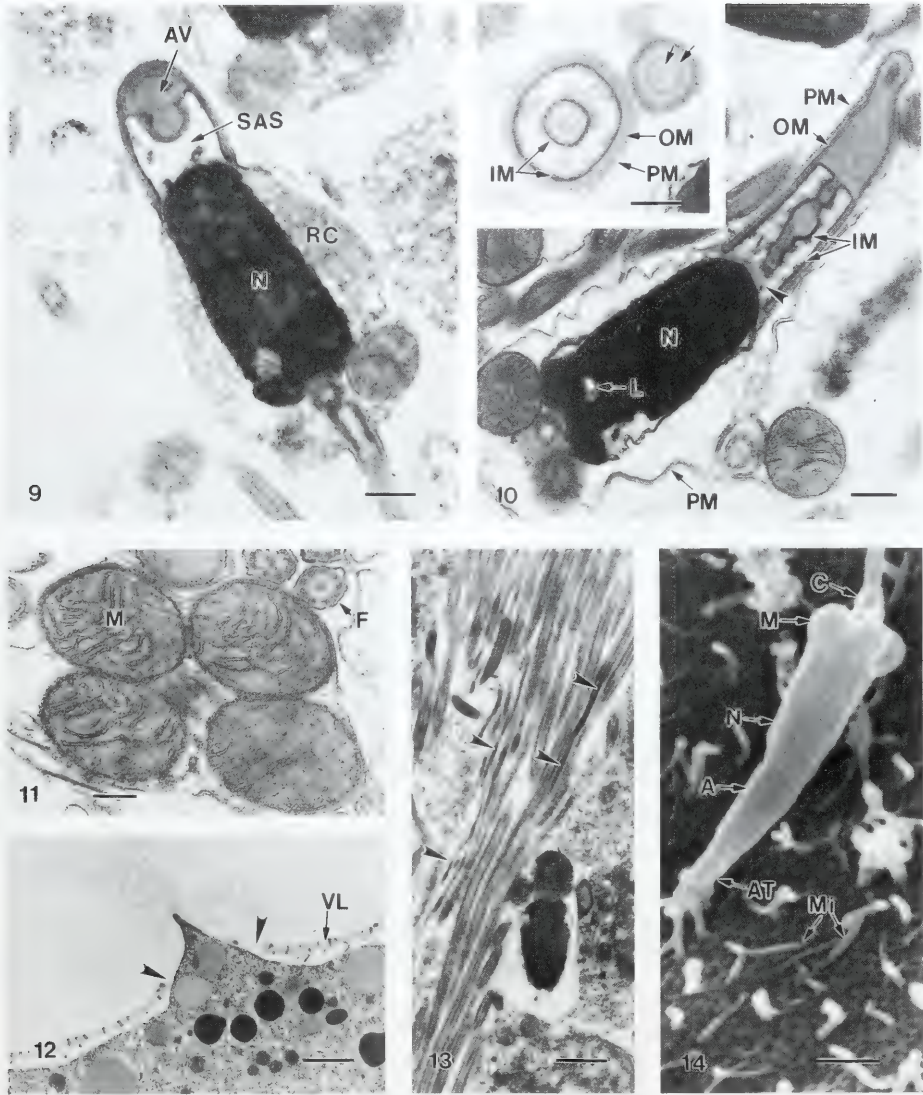
Figure 4. Longitudinal section of spermatid with proacrosomal vesicle (PAV) migrating to presumptive anterior of cell. Note Flagellum (F) and cytoplasmic bridges (*) connecting adjacent spermatids of a cohort. Scale bar = 0.5 μm .

Figure 5. Spermatid acrosomal vesicle (AV) with cup-shaped morphology at apex of nucleus. Proximal centriole (PC), distal centriole (DC), mitochondrion (M), and electron-dense nuclear fossa (arrowhead). Scale bar = 0.5 μm .

Figure 6. Cup-shaped acrosomal vesicle (AV) above nucleus (N) with circular profile of possible small proacrosomal vesicle (arrowhead) in subacrosomal space. Scale bar = 0.4 μm .

Figure 7. Later spermatid than in Fig. 6, showing elongating nucleus (N) and lateral extension of acrosomal vesicle (arrowheads). Proximal centriole (PC) is attached to nuclear fossa; distal centriole (DC) is harnessed to plasma membrane by the annulus (An) via the centriolar satellite complex. Scale bar = 0.5 μm .

Figure 8. Spermatid showing acrosomal vesicle forming posterior lobe (arrowhead). Note condensation of granular chromatin (GC) into large granules. Scale bar = 0.5 μm .



Figures 9–14. Electron micrographs of sperm and egg structure in *Tectura testudinalis*.

Figure 9. Longitudinal section of late spermatid. Acrosomal vesicle (AV) extending posterior lobe into subacrosomal space (SAS). Residual cytoplasm (RC) next to nucleus (N) will be shed when sperm matures. Scale bar = 0.4 μ m.

Figure 10. Longitudinal section through mature sperm showing plasma membrane (PM) enclosing outer (OM) and inner (IM) acrosomal membranes. Note nucleus (N) with lacuna (L), acrosome with layered contents and subacrosomal flocculent material (arrowhead). Scale bar = 0.4 μ m. **Inset:** Transverse sections of acrosome near tip, showing electron-dense stratifications (small arrows) and through subacrosomal space showing outer (OM) and inner (IM) acrosomal membranes enclosed by plasma membrane (PM). Scale bar = 0.3 μ m.

arrangement of microtubules in the principal-piece (Fig. 11) and tapers into a filamentous electron-dense end-piece (Fig. 13). These observations are summarized diagrammatically in Figure 27.

Surface ultrastructure of unfertilized egg

Intact unfertilized eggs are characterized by two distinct egg envelopes (Figs. 15–18). The outermost envelope is a discontinuous jelly coat about 3 μm thick. This jelly coat surrounds a very thin (0.3 μm), transparent vitelline layer that overlies the plasma membrane. Numerous microvillous extensions of the egg plasma membrane (0.3 μm or more in length) are embedded in the vitelline layer, resulting in small bumps or protrusions of the vitelline layer above the egg surface (Figs. 17, 24–26). Moreover, spaced regularly over the egg surface are a series of much larger, more elongate microvilli (up to 3 μm in length), which penetrate through the vitelline layer and extend up into the egg jelly coat (Fig. 15, 16). Follicle cells overlying the egg jelly coat are connected to the oocyte by microvillous extensions that form junctions with the elongate egg microvilli (Fig. 16). These connections appear at regular intervals over the egg surface (Fig. 15) and appear to disrupt the vitelline layer at these points (Fig. 12). In some areas where the follicle cell processes have retracted, the egg jelly diffuses into the surrounding medium (Figs. 15, 17). Thus, the egg jelly coat of freshly spawned eggs is sometimes discontinuous, where it has dissolved between retracted follicle cells. Furthermore, the jelly coats of adjacent eggs may become stuck to one another, producing small aggregates of eggs.

Early events of fertilization

Mature sperm, in attempts to fertilize the egg, often get lodged in the thick jelly coat (Figs. 18, 18 inset). Sperm in contact with the jelly coat do not undergo an acrosome reaction involving exocytosis of the acrosomal vesicle, but in many instances they do undergo an elongation of the anterior portion of the acrosome (Figs. 18, 19, 22). The elongated acrosome tip is characterized by longitudinal mi-

crofilaments, which were not present before elongation (Figs. 19, 19 inset). Posterior to the elongating tip in the middle and base of the acrosome, there appear small membranous vesicles (Fig. 19). During this elongation, the ring of spherical mitochondria usually become compacted into more oblong structures (Figs. 25, 26).

Upon penetration of the jelly coat, the tip of the acrosome comes in contact with the vitelline layer. Contact with the vitelline layer overlying the microvilli initiates the typical acrosome reaction, in which the outer acrosomal membrane and plasma membrane fuse at the apex and roll back in a manner that releases the contents of the acrosomal vesicle onto the vitelline layer (Figs. 25, 26). Following this, the posterior lobe of the inner acrosomal membrane everts and protrudes from within the acrosome (Figs. 25, 26). It is probably during this process that the vitelline layer is dispersed and the inner acrosomal membrane is brought into contact with one or more exposed microvilli of the egg plasma membrane. However, primary contact may also occur between the larger, naked egg microvilli that formerly connected with the follicle cell processes (Figs. 24, 25).

Contact between a naked egg microvillus and the inner acrosomal membrane results in fusion and the creation of a tube through which the nucleus and other sperm organelles gain access to the egg cytoplasm. Following this initial fusion, other microvilli become attached to the inner acrosome, including the larger microvilli that formerly anchored the follicle cells (Fig. 25). The combined fusion of these microvilli results in the production of a fertilization cone, which engulfs the fertilizing sperm. Cortical granules in the cortex of the egg are released following sperm-egg fusion and presumably are involved in a block to polyspermy, as in other invertebrates. On several occasions, follicle cells that had phagocytized supernumerary sperm were found on the surface of fertilized eggs (Fig. 23).

Discussion

Spermiogenesis and ultrastructure of mature sperm

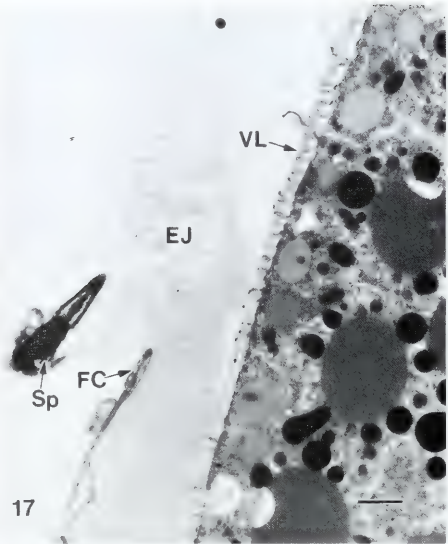
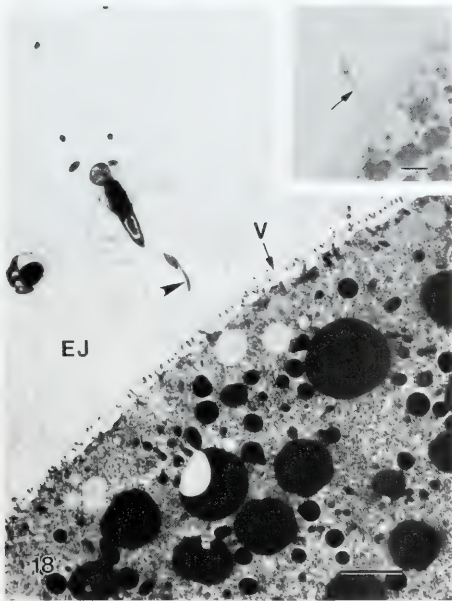
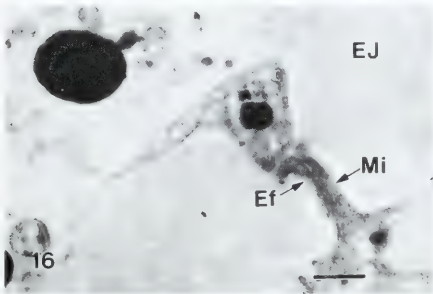
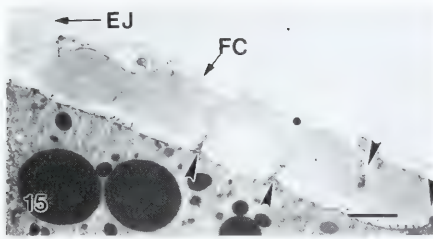
The structure of the unreacted acrosome is similar to that described for some Patellidae (Hodgson and Bernard,

Figure 11. Transverse section of mid-piece of mature sperm showing ring of four spherical mitochondria (M). Note adjacent flagellum (F) with 9 + 2 arrangement of microtubules. Scale bar = 0.2 μm .

Figure 12. Section perpendicular to egg surface showing portion of elongate microvillus penetrating otherwise intact vitelline layer (arrowheads) to make connection with follicle cell process (not shown). Scale bar = 2.0 μm .

Figure 13. Longitudinal section through flagella tapering to electron-dense filamentous end-pieces (arrowheads). Scale bar = 1.0 μm .

Figure 14. Scanning electron micrograph of mature sperm. Note relative proportions of mature sperm components, including acrosome (A) with distinct tip (AT), nucleus (N), spherical mitochondria (M), and collar (C) surrounding flagellum. Note few large egg microvilli (Mi) that formerly bound the follicle cells above the jelly coat; many small microvilli beneath vitelline layer make pattern of bumps on egg surface. Scale bar = 1.0 μm .



Figures 15–19.

Figure 15. Follicle cells (FC) connected to oocyte membrane at regular intervals over egg surface (arrowheads). Note dissipation of egg jelly coat (EJ). Scale bar = $3.0\ \mu\text{m}$.

Figure 16. Interdigitation of elongate microvilli (Mi) from oocyte membrane with extension of follicle cell (Ef) within egg jelly coat (EJ). Scale bar = $1.0\ \mu\text{m}$.

1988; Hodgson, 1995) but is closest to that of the Lottiidae (Hodgson and Chia, 1993), all of which are characterized by an acrosomal vesicle with an invaginated posterior lobe that protrudes into the subacrosomal space. However, in *Helcion*, preformed microfilaments in the anterior acrosome project down into the posterior lobe, deep into the subacrosomal space (Hodgson and Bernard, 1988). These microfilaments are unusual in having a diameter of 9 nm, which neither matches actin (6 nm) nor intermediate filaments (10 nm) in size. Similarity in sperm structure between species of *Lottia* and *Tectura* provides support for Lindberg's (1986) reclassification of all *Acmaea* (except *A. mitra*) and all *Notoacmea* (except Australian and New Zealand species) into the genus *Tectura* within the Lottiidae. The structural changes that occur during spermiogenesis in *Tectura testudinalis* are similar to those described for other Lottiidae (Hodgson and Bernard, 1988; Sousa and Oliveira, 1994; Hodgson, 1995). However, the formation of an acrosomal vesicle from fusion of smaller Golgi-derived vesicles, confirmed in this study, has been demonstrated previously only in *Helcion pellucidus* (Sousa and Oliveira, 1994). Moreover, in our study, proacrosomal vesicle formation occurs in the spermatid stage, whereas Sousa and Oliveira (1994) report its occurrence in the primary spermatocyte. In the paper by Sousa and Oliveira (1994), cells described as primary spermatocytes appear to be spermatids, based on the appearance of chromatin (figures 3 and 4) and the position and presence of centrioles associated with mitochondria (figure 4). Regardless, this type of proacrosome formation is consistent with similar processes reported for other archaeogastropods (Azevedo *et al.*, 1985; Koike, 1985; Buckland-Nicks and Chia, 1986) and bivalves (Longo and Dornfeld, 1967), and it is probably present throughout the Patelloidea. It contrasts with the development of the acrosome in caenogastropods, and other invertebrates, from a single cisterna of the Golgi body which remains connected throughout development (Buckland-Nicks and Chia, 1976; Healy, 1982; Koike, 1985).

The typical flagellum of *T. testudinalis* tapers to a filamentous end-piece less than one-third of its diameter. This has only once been reported in Patelloidea (Azevedo,

1981), but it also occurs among Aplacophora and Polyplacophora (Buckland-Nicks, 1995), as well as some Caenogastropoda (Giusti and Mazzini, 1973). It is possible that the filamentous end-piece has not been noticed previously in other patellids and archaeogastropod groups, and may be a useful character in phylogenetic analysis.

Surface ultrastructure of unfertilized eggs

The discontinuous nature of the jelly coat of some *T. testudinalis* eggs suggests that its role is not to act as a sperm barrier, nor to induce the typical acrosome reaction in which the acrosomal vesicle releases its contents, but rather to serve some other role in producing successful fertilization. The egg jelly coat of some keyhole limpets has been shown to contain sperm-activating agents (Tyler and Fox, 1940). Retraction of the follicle cells, which exposes the egg jelly coat in *T. testudinalis*, may result in the release of sperm-activating and sperm-attracting substances as the egg jelly coat dissipates. In thick sections, a relatively high number of sperm were found associated with egg jelly coat bursts from the follicle cell layer.

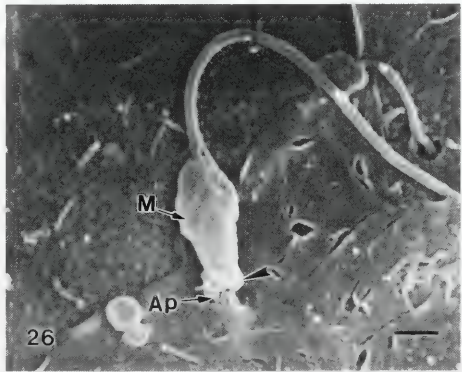
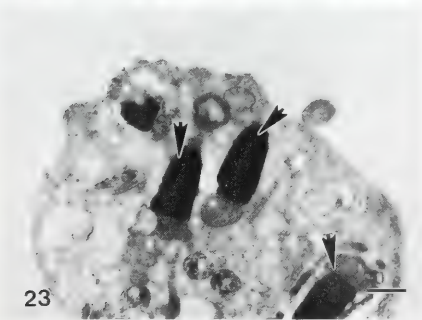
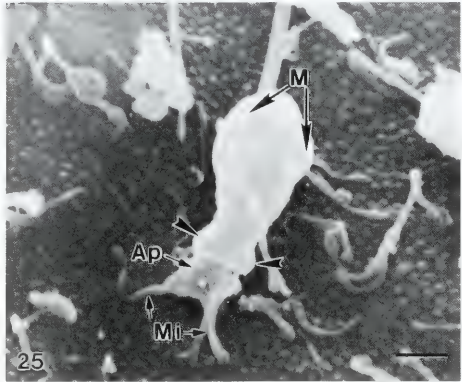
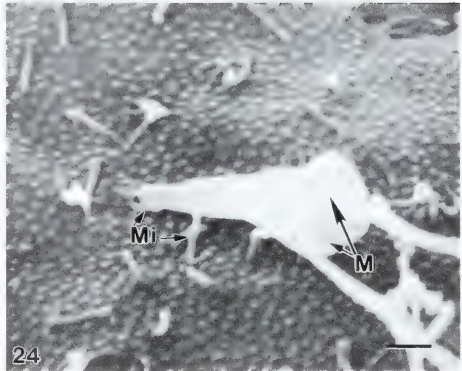
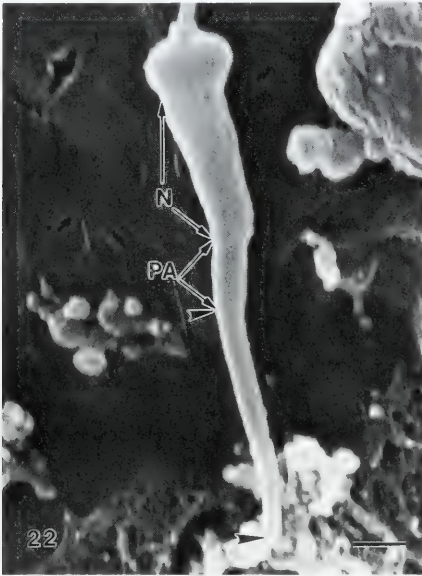
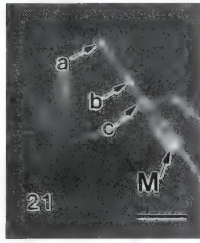
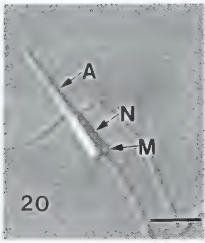
The egg jelly coats of neighboring eggs often stick together to produce an aggregate of eggs. Similar aggregates have been noted in other molluscan groups (Eernisse, 1988; Buckland-Nicks, 1995). Studies by Levitan (1993) on echinoderms have shown that larger eggs may present a larger target for sperm and thus become fertilized at a higher rate. Perhaps aggregates of eggs mimic a larger egg target. Selective pressure would favor such aggregates if they resulted in relatively higher fertilization rates than for individual eggs.

It was surprising to find that follicle cells had assumed a phagocytic function on the surface of fertilized eggs. However, on several occasions, sperm were found inside follicle cells in an obvious state of degeneration. Do waste sperm in some way pose a threat to the zygote? What is the fate of these follicle cells? In other molluscs, such as chitons, the follicle cells are shed before spawning or soon afterwards, because in many species they cover receptive sites on the egg hull (Buckland-Nicks, 1995).

Figure 17. Section of egg showing approaching sperm (Sp), dissipating egg jelly coat (EJ), and vitelline layer (VL) overlying plasma membrane. Note retracted follicle cell process (FC). Scale bar = 1.5 μ m.

Figure 18. Transmission electron micrograph showing sperm in egg jelly coat (EJ). Note thin vitelline layer (V) and elongated anterior tip of acrosome (arrowhead) belonging to adjacent sperm. Scale bar = 3 μ m. **Inset:** Light micrograph of 1- μ m section of sperm with elongate acrosome (small arrow) penetrating egg jelly coat. Scale bar = 4.0 μ m.

Figure 19. Transmission electron micrograph of a section of elongated anterior tip of acrosome (AT) in egg jelly coat (EJ). Note membranous vesicles (MV) in acrosome; posterior lobe (arrow) still protrudes into subacrosomal space, indicating lack of acrosome reaction. Scale bar = 0.4 μ m. **Inset:** Microfilaments (small arrows) in elongated anterior tip of acrosome. Scale bar = 0.1 μ m.



Figures 20–26.

Figure 20. Nomarski light micrograph of mature sperm of *Tectura scutum*, showing acrosome (A) nucleus (N) and mitochondrion (M). Scale bar = 2.0 μ m.

Figure 21. Same sperm as in Fig. 20, labeled with anti-actin antibody conjugated to FITC, showing three regions of actin storage in the unreacted acrosome. Acrosome tip (a), anterior to subacrosomal space

Early events of fertilization

The acrosome of *T. testudinalis* appears to be involved in several key functions during fertilization, including (1) elongation of the anterior tip of the acrosome following contact with the egg jelly coat, (2) conversion of membrane precursors into new membrane to enable this elongation, (3) digestion of egg envelopes prior to sperm-egg fusion.

Acrosome elongation. Sperm association with the egg jelly coat triggers an elongation of the anterior tip of the acrosome in some sperm of *T. testudinalis*. This event is distinct from that in sea urchins and starfishes, where the jelly coat and its constituent compounds trigger an acrosome reaction (Epel, 1978; Hoshi *et al.*, 1994). Elongation of the anterior tip of the acrosome is distinct from an "acrosome reaction," as there is no eversion of the inner acrosomal membrane or protrusion of the subacrosomal space by an acrosomal process (Sato and Osanai, 1983). Micrographs of the anterior acrosome extension of *T. testudinalis* in the egg jelly coat suggest the presence of 6-nm microfilaments that were not apparent in the mature sperm before elongation. Azevedo made similar observations in *Patella lusitanica* (1981) and in the vetigastropod *Gibbula umbilicalis* (Azevedo *et al.*, 1985), reporting an extension of the anterior tip of the acrosome in response to egg water. Furthermore, in the related species *T. scutum*, we found actin localized at the tip, middle, and posterior of the acrosome (Figs. 20, 21). On the basis of these findings, we suggest that the newly formed microfilaments in the anterior tip of the acrosome of *T. testudinalis* are probably a result of polymerization of G-actin in the tip of the sperm acrosome, triggered by some property of the egg jelly coat. The mechanism of acrosome extrusion may vary among patellids, as evidenced by the report of pre-existing 9-nm filaments in the anterior acrosome of *Helcion* (Hodgson and Bernard, 1988). In other respects, the acrosome of these patellids is very similar to that of *T. testudinalis*.

We propose that the extension of the anterior tip of the

acrosome has evolved to penetrate the egg jelly coat, when present. Although this elongation of the acrosome is not necessary for fusion of sperm and egg, it may provide a sperm with a selective advantage in penetrating the intact jelly coat of freshly spawned eggs. The egg jelly coat, besides functioning to stimulate extension of the acrosome in *Tectura*, may have other properties—including release of sperm chemoattractants (Miller, 1985) or species-specific sperm-activating agents (Tyler and Fox, 1940)—found in other patellids.

New membrane from precursors. The elongation of the acrosomal tip occurs within the limits of the plasma membrane. Consequently, any folds in the membrane are stretched out and the spherical mitochondria become compacted. Additional membrane may be developed from membrane precursors, as suggested previously for echinoderms (Dan, 1967; Colwin *et al.*, 1975; Sardet and Tilney, 1977; Tilney, 1985). Small membranous vesicles were found in elongating acrosomes in *T. testudinalis* but not in mature sperm prior to elongation. These vesicles apparently formed spontaneously during acrosome elongation and may be responsible for producing additional membrane by exocytosis. A very similar process, in which membranous vesicles formed spontaneously and contributed new membrane to the acrosomal process (Colwin *et al.*, 1975), was described in the fertilization of a holothurroid. Likewise, when sperm of *Gibbula umbilicalis* were exposed to seawater containing oocytes, several small vesicles were seen differentiating in the apical zone of the acrosome (Azevedo *et al.*, 1985).

The acrosome reaction and eversion of the inner acrosomal membrane. Following penetration of the egg jelly coat, sustained contact of the anterior tip of the acrosome with the vitelline layer overlying the small egg microvilli induces the acrosome reaction and subsequent fusion with the egg membrane. However, the larger, naked microvilli may supersede this process by reacting directly with the acrosome tip, or they may become involved subsequently by binding to the anterior portion of the acrosome.

(b), and subacrosomal space (c). Other sites of actin in the sperm are mitochondrial region (M) and flagellum. Scale bar = 2.0 μ m.

Figure 22. Scanning electron micrograph showing extended acrosome tip (between arrowheads). Note position of nucleus (N) and posterior half of acrosome (PA). Scale bar = 1.0 μ m.

Figure 23. Transmission electron micrograph of follicle cell containing sperm in heterophagic vacuoles in cytoplasm (arrows). Scale bar = 1.3 μ m.

Figure 24. Scanning electron micrograph of sperm acrosome tip fusing with elongated egg microvilli (Mi). Note mitochondria (M). Scale bar = 1.0 μ m.

Figure 25. Sperm acrosome reaction. Elongate egg microvilli (Mi), acrosome process (Ap), rolling back of plasma and outer acrosomal membranes (arrowheads), and compacted mitochondria (M). Scale bar = 1.0 μ m.

Figure 26. Late stage of sperm entry through jelly coat, showing extended acrosomal process (Ap), rolling back of acrosome and plasma membranes (arrowhead), and compacted mitochondria (M). Scale bar = 1.0 μ m.

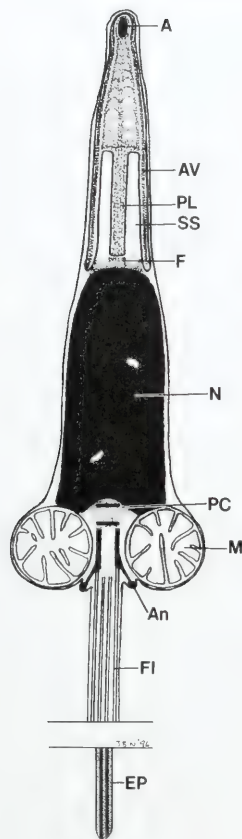


Figure 27. Diagram of mature sperm of *Tectura testudinalis*. Note: site of actin in acrosomal tip (A), acrosomal vesicle (AV), subacrosomal space (SS), posterior lobe (PL), flocculent material of subacrosomal space (F), nucleus (N), proximal centriole (PC), distal centriole and centriolar satellite connectives attached via annulus (An) to plasma membrane, mitochondrion (M), flagellum (FI), and dense end-piece (EP) with reduced microtubules.

One key difference between *T. testudinalis* and other molluscan groups is the nature of the acrosomal process that extrudes the inner acrosomal membrane. In some vetigastropods, flocculent material located in the subacrosomal space is responsible for everting the inner acrosomal membrane by polymerization to a filamentous axial rod (Azevedo *et al.*, 1985). In other invertebrate species, including many echinoderms, arthropods, and annelids (see review by Dan, 1967), an acrosomal process is extruded from the subacrosomal space after polymerization

of fibrogranular material into a microfilamentous rod. This contrasts with the mechanism observed in other molluscs—including some archaeogastropods (Lewis *et al.*, 1980) and bivalves (Tilney, 1985; Tilney *et al.*, 1987)—in which intersliding of actin filaments arranged in pre-formed rods causes extension of the acrosomal process.

Exactly how the inner acrosomal membrane everts in *T. testudinalis* is not known, but a similar polymerization of the flocculent fibrogranular material in the subacrosomal space by an actin-based mechanism is likely, considering that this site was one of three where actin was located in the related species, *T. scutum*. The final result is the extension of a short, broad acrosomal process, which contacts the egg microvilli and results in sperm-egg fusion. This type of acrosome reaction, as well as subsequent sperm entry into the egg, is very similar to that described for the polychaete *Tylorrhynchus heterochaetus* (Sato and Osanai, 1983; compare their fig. 6, p. 466, to our Fig. 25), as well as the scaphopod *Dentalium vulgare* (Dufresne-Dubé *et al.*, 1983) but is unlike the acrosome reaction and sperm entry described for other archaeogastropods (Lewis *et al.*, 1980; Shiroya and Sakai, 1984; Azevedo *et al.*, 1985).

Phylogenetics

In his analysis of gastropod phylogeny, Haszprunar (1988) contended, on the basis of characters such as gill structure, external fertilization with aquasperm, and symmetrical shell form, that docoglossan limpets (including Patellidae) are the basal offshoot of the ancestral gastropod. However, several authors have argued that almost none of these characters are primitive (see Ponder and Lindberg, 1996) and that in general "patellogastropods have few characters that match those seen in early Palaeozoic gastropod-like fossils" (Runnegar, 1996). Buckland-Nicks and Scheltema (1995) proposed that internal fertilization, not external, is basal to the Bilateria; evidence for this conclusion came from analysis of similar introsperm design in primitive members of vertebrate and invertebrate phyla. Among molluscs, the Neomeniomorpha, which have internal fertilization, are currently regarded as basal to the Mollusca in light of recent molecular and morphological analyses (Runnegar, 1996; Scheltema *et al.*, 1994). Thus, external fertilization does not necessarily denote primitive status among bilaterian phyla, although it is probably archetypal to the cnidarians and sponges.

Fertilization in limpets appears to be specialized in a number of ways, including the structure of the egg envelopes and the association with follicle cells; the use of a mechanism of sperm penetration that involves elongation of the acrosome tip prior to the acrosome reaction; and the expulsion of a short, broad acrosomal process. Furthermore, analysis of sperm basic proteins of limpets and

other gastropods indicated "a clear divergence of the protamines of *Patella* and other archaeogastropods" (Daban *et al.*, 1990, p. 127). This information and the above discussion suggest that it is premature to assign basal status to the Docoglossa. Rather, there is growing support for viewing limpets as a divergent group (Ponder and Lindberg, 1996) whose ancestors may have had internal fertilization with intospERM, a helicoid larval shell (Page, 1994), and primitive ctenidia.

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Hermaphroditic Freshwater Clams in the Genus *Corbicula* Produce Non-Reductional Spermatozoa With Somatic DNA Content

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Abstract. Hermaphroditic freshwater clams in the genus *Corbicula* produce non-reductional spermatozoa. The DNA content of spermatozoa was almost identical with that of somatic cells in *C. leana* from Mie Prefecture, Japan. Hermaphroditic *C. aff. fluminea* from Saga Prefecture and *C. fluminea* from Taiwan also produce non-reductional spermatozoa. On the other hand, spermatozoa of the dioecious *C. sandai* had half the DNA found in somatic cells. Analysis of chromosome numbers suggests that *C. leana* ($3n = 54$ in somatic cells and 18 in meiotic cells) from Mie Prefecture and *C. aff. fluminea* ($2n = 36$ in gills and 18 bivalents in meiotic cells) from Saga Prefecture are triploids and diploids, respectively. *C. leana*, *C. aff. fluminea*, and *C. fluminea* may lack either first or second meiosis, resulting in non-reductional spermatozoa. We assume that gynogenetic reproduction occurs in both species; maternal chromosomes are also non-reductional, and spermatozoa activate development of the eggs, but do not contribute to the offspring.

Introduction

The freshwater clam *Corbicula leana* has a special mode of reproduction: it is hermaphroditic and broods its larvae in the inner demibranchs (Miyazaki, 1936; Ike-matsu and Yamane, 1977). A recent study of the chromosomes of *C. leana* showed that it has 54 somatic chromosomes, which, judging from its karyotype, are triploids

(Okamoto and Arimoto, 1986). Okamoto and Arimoto (1986) suggested the possibility that *C. leana* reproduces by gynogenesis, which would account for the odd chromosome number: i.e., gynogenetic eggs are usually meiotically unreduced, and spermatozoa activate the development of eggs, but the paternal genome does not contribute to the offspring. Analysis of allozyme variation in *C. leana* also revealed that the species has much lower genetic variability than *C. japonica* and *C. sandai* (Sakai *et al.*, 1994). In this study, we compare chromosome numbers and DNA content of somatic cells and spermatozoa in the hermaphroditic *Corbicula* species to test the gynogenesis hypothesis proposed by Okamoto and Arimoto (1986).

Materials and Methods

The *Corbicula* species were identified according to Habe (1977). *C. leana* and *C. aff. fluminea* were collected in Japan from an irrigation canal in Meiwa, Mie Prefecture, and from the Tade River, Saga Prefecture, respectively. In this study we use the name *C. aff. fluminea* for clams collected from Saga Prefecture because the morphology of the lateral teeth in these specimens was very similar to that of *C. fluminea* (Komaru *et al.*, 1998). *C. fluminea* was also obtained from a cultured population in Shin Wu, Taiwan. For comparison using microfluorometry, specimens of *C. sandai* from Lake Biwa, Shiga Prefecture, were obtained at the market in Ootsu city.

Chromosomes

The clams were treated with 0.002% colchicine for 4–5 h. Gills and gonads were dissected out with scissors,

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Figure 1. Mitotic (A, C) and meiotic (B, D, E) chromosomes of *Corbicula leana* from Mie Prefecture (A, B) and *C. aff. fluminea* from Saga Prefecture (C, D, E). Scale bar = 10 μ m.

treated with hypotonic solution (8 mM KCl), and fixed with Carnoy's fixative according to the procedure of Okamoto and Arimoto (1986). Cells were isolated from either gill or gonads, placed on slides, and stained with 2% Giemsa in phosphate buffer (pH 7.2). At least 4 clams were examined for each case (*i.e.*, each combination of species and tissue type).

DNA microfluorometry

To compare the relative DNA content of spermatozoa and somatic tissue, cells were isolated on a glass slide by cutting a small piece of gonad or mantle in distilled water with a scalpel and air-drying it before fixing it with 70% ethanol. Spermatozoa and somatic cells from one individual were placed on the same slide. The cells were stained with the DNA-specific dye DAPI, and the relative DNA content (fluorescence intensity) per cell was estimated by microfluorometry as in Komaru *et al.* (1988). Spermatozoa could be easily distinguished from other sperm-

atogenic cells because of their elongate and curved morphology.

Results

Mitotic and meiotic chromosomes

In four specimens of *C. leana*, we counted 54 chromosomes in the mitotic metaphase plates of somatic tissue (Fig. 1A) and about 18 chromosomes in the gonad (Fig. 1B). In *C. aff. fluminea*, we observed 36 chromosomes in the mitotic metaphase in 16 cells, but the haploid number of 18 in 8 gonadal cells. Pachytene (Fig. 1D), diakinesis or metaphase I (Fig. 1E) figures were observed in the meiotic cells.

Relative DNA content of spermatozoa and somatic cells

In *C. leana*, *C. aff. fluminea*, and *C. fluminea*, the relative content of DNA in the spermatozoa was also identical

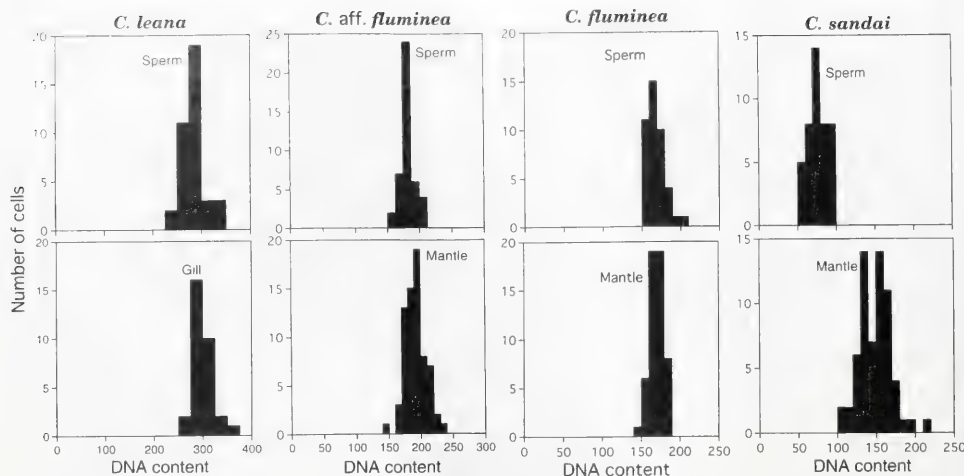


Figure 2. Histograms of fluorescence intensity (relative DNA content) in spermatozoa and somatic cells of *Corbicula leana* from Mie Prefecture, *C. aff. fluminea* from Saga, *C. fluminea* from Taiwan, and *C. sandai* from Shiga. Each histogram was derived from one individual. DNA content per cell was measured as fluorescence intensity.

to that in the somatic cells (Fig. 2. and Table I). In *C. sandai*, however, the DNA content of the spermatozoa was half that of the somatic cells.

Discussion

Okamoto and Arimoto (1986) reported chromosome numbers of $3n = 54$ for *C. leana*, $2n = 38$ for *C. japonica*,

and $2n = 36$ for *C. sandai*. In the present study, the chromosome number of *C. aff. fluminea* was determined to be $2n = 36$ in gill tissue and 18 bivalents in gonads, indicating that *C. fluminea* is a diploid species. In contrast, *C. leana* has a somatic chromosome number of 54, but in the gonads, three homologous chromosomes form 18 trivalents. The synapsis of three homologous chromosomes should be incomplete. These results suggest that *C. leana* is a triploid species.

The microfluorometric measurements of DNA revealed that the hermaphroditic species *C. leana* and *C. fluminea* produced non-reductional spermatozoa; i.e., sperm and somatic cells had the same DNA content. On the other hand, the dioecious *C. sandai* produced reductional spermatozoa; the DNA content of sperm was half that of somatic cells. These results suggest that the cytokinesis of first or second meiosis in the spermatocytes of both *C. leana* and *C. fluminea* is abortive, and only one equal division occurs. Consequently, meiosis during spermatogenesis may be non-reductional, resulting in spermatozoa with somatic ploidy levels.

It is possible that triploid *C. leana* and diploid *C. fluminea* reproduce by gynogenesis, as O'Foighil and Thiriot-Quievreux (1991) observed in the bivalve *Lasaea*. In gynogenetic development, spermatozoa stimulate the development of eggs but do not contribute paternal genome to the offspring. In this case the somatic chromosome number may be restored to the eggs by premeiotic endomitosis

Table I

Relative DNA content of spermatozoa and somatic cells in three species of *Corbicula*

Species	Sample no.	DNA content (mean $\pm$ SD)	
		Somatic cell	Spermatozoa
<i>C. leana</i>	1	251.6 $\pm$ 23.6 (n = 94)	251.2 $\pm$ 24.8 (n = 53)
	2	259.5 $\pm$ 17.4 (n = 66)	278.5 $\pm$ 45.2 (n = 16)
	3	295.0 $\pm$ 25.4 (n = 119)	328.5 $\pm$ 29.0 (n = 26)
	4	315.5 $\pm$ 32.6 (n = 28)	317.9 $\pm$ 36.6 (n = 44)
<i>C. aff. fluminea</i>	1	192.4 $\pm$ 17.6 (n = 70)	181.9 $\pm$ 12.5 (n = 23)
	2	176.0 $\pm$ 10.8 (n = 22)	175.5 $\pm$ 18.9 (n = 24)
	3	204.0 $\pm$ 18.3 (n = 48)	203.8 $\pm$ 10.1 (n = 48)
	4	183.7 $\pm$ 13.5 (n = 39)	179.6 $\pm$ 13.1 (n = 56)
<i>C. fluminea</i>	1	160.6 $\pm$ 12.1 (n = 27)	161.0 $\pm$ 13.3 (n = 26)
	2	172.4 $\pm$ 12.9 (n = 53)	191.2 $\pm$ 13.7 (n = 26)
	3	151.1 $\pm$ 7.9 (n = 54)	168.5 $\pm$ 10.5 (n = 34)
	4	161.3 $\pm$ 11.2 (n = 41)	166.0 $\pm$ 9.7 (n = 40)
<i>C. sandai</i>	1	202.8 $\pm$ 12.8 (n = 36)	109.3 $\pm$ 6.4 (n = 38)
	2	164.1 $\pm$ 14.5 (n = 53)	90.3 $\pm$ 8.2 (n = 38)
	3	148.3 $\pm$ 20.0 (n = 63)	75.7 $\pm$ 12.2 (n = 38)
	4	180.8 $\pm$ 11.3 (n = 34)	99.3 $\pm$ 15.3 (n = 16)

Each mean value and standard deviation (SD) was derived from one individual. The numbers in parentheses (n) are the number of cells measured.

or abortive meiosis (Cuellar, 1987). How did the triploid *C. leana* evolve from a diploid ancestral species? We assume that the diploid gynogenetic *C. fluminea* arose first. Later, the triploid *C. leana* may have evolved from the diploid gynogen by fusion of diploid and haploid gametes. The processes of meiosis and fertilization of eggs should be observed in *C. leana* and *C. fluminea* to understand the reproductive processes in these species with non-reductional spermatozoa.

Species of *Corbicula* show polymorphic shell color and morphology (Kuroda, 1938; Morton, 1986). The taxonomy of the Corbiculidae is in disarray as a result of intra-specific variation in shell morphology and insufficient biological information. *Corbicula* taxonomy cannot be clarified on the basis of shell morphology alone; as Morton (1986) suggested, ecological, genetic, and physiological studies will also be necessary. If the genus *Corbicula* includes polyploid species that reproduce by gynogenesis, as our results indicate, it will be difficult to apply the biological species concept to these clams (Mayr and Ashlock, 1991). The definition of species in *Corbicula* should be addressed after sufficient data on reproductive biology have been collected.

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Oocyte Maturation in the Brachiopod *Terebratalia transversa*: Role of Follicle Cell-Oocyte Attachments During Ovulation and Germinal Vesicle Breakdown

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Abstract. In the brachiopod *Terebratalia transversa*, each ovarian follicle consists of (i) a prophase-arrested oocyte with an enlarged nucleus (= the germinal vesicle, or GV), and (ii) a surrounding sheath of follicle cells that attach to the oocyte by means of junctional complexes. Within about 80 min after removing a follicle from the ovary, the follicular sheath undergoes a microfilament-mediated retraction, and the ovulated oocyte that emerges from the retracted sheath subsequently completes germinal vesicle breakdown (GVBD). Based on the experimental manipulations reported here, it appears that the follicle must be detached from the ovary for such ovulation and GVBD to occur. Moreover, GVBD can be prevented if the oocytes are mechanically stripped of their follicle cells up to 30 min after being isolated from the ovary. GVBD proceeds normally, however, if follicle cells are removed more than 40 min after the follicle is obtained from the ovary. The percentage of oocytes that undergo GVBD is also diminished following treatment with drugs that uncouple gap junctions. Collectively, these data suggest that removing a follicle from the ovary stimulates follicle cells to produce a maturation-inducing factor that uses the follicle cell–oocyte junctional complexes to reach the oocyte within about 30–40 min after follicular removal. The significance of these findings is discussed relative to previous reports on oocyte maturation in brachiopods and other animals.

Introduction

In many groups of animals, oocytes develop in close association with a surrounding sheath of follicle cells as part of an integrated unit called a follicle (Raven, 1961).

During differentiation of a typical follicle, the oocyte nucleus (= the germinal vesicle, or GV) becomes greatly enlarged while the oocyte remains arrested in prophase I of meiosis. In addition, projections of the follicle cells also can attach to the oolemma of the oocyte by means of gap junctions that allow small molecules to pass between the follicle cells and the prophase-arrested oocyte (Anderson and Albertini, 1976; Patino and Purkiss, 1993; York *et al.*, 1993). When triggered by the proper stimulus, the oocyte resumes meiotic maturation by initiating germinal vesicle breakdown (GVBD) and ultimately emerges from its follicular sheath during a process referred to as ovulation.

As noted in various investigations (Eppig, 1985; Kwon *et al.*, 1990; Schultz, 1991; Downs, 1997), maturation depends on a dynamic interplay between oocytes and their surrounding follicle cells. For example, follicle cells can secrete maturation-inducing substances that presumably use the junctions between follicle cells and oocytes to promote GVBD and ovulation (Schroeder, 1981; Beers and Olsiewski, 1985; Sato and Koide, 1987; Cerada *et al.*, 1993). On the other hand, mammalian follicle cells may initially produce substances that inhibit maturation; this conclusion is based on the observation that competent oocytes undergo spontaneous maturation if removed from their follicular sheaths (Pincus and Enzmann, 1935) and on subsequent confirmations of follicle-cell-derived inhibition in experimental analyses (Cho *et al.*, 1974; Racowsky, 1985). More recent investigations, however, suggest that mammalian follicle cells also supply a maturation-inducing substance that overcomes the effects of the previous inhibition of maturation (Downs *et al.*, 1988; Downs, 1995).

Thus, follicle cells can stimulate or inhibit maturation, and such regulatory signals may be transported through the junctions at the follicle cell–oocyte interface. To supplement our current understanding of the roles of follicle

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cells during oocyte maturation, additional studies of a broader range of animals are needed.

Brachiopods constitute a comparatively small phylum of sessile or sedentary marine invertebrates that possess a calcified shell and a ciliated lophophore used in filter feeding (Hyman, 1959; Williams and Rowell, 1965; Rudwick, 1970). The roughly 120 genera of living brachiopods are traditionally grouped into two classes—the Inarticulata and Articulata—which are distinguished on the basis of differences in shell structure, anatomy, and reproductive biology (James *et al.*, 1992).

Recent reviews of brachiopod reproduction and embryology provide relatively little information about oocyte maturation (Chuang, 1983; Reed, 1987; Long and Stricker, 1991). Since those reviews were published, thorough investigations have been conducted on the process of oogenesis in the articulate brachiopod *Terebratulina retusa* (James *et al.*, 1991a, b; 1992) and on the endocrine pathway leading to oocyte maturation in the inarticulate species *Glottidia pyramidata* (Freeman, 1994). However, neither the precise relationship between ovulation and GVBD nor the possible roles played by follicle cell–oocyte attachments during maturation have yet been defined for brachiopods.

In the investigation reported here, time-lapse video microscopy, electron microscopy, and experimental manipulations were used to analyze *in vitro* oocyte maturation in the articulate brachiopod *Terebratalia transversa*. The results suggest that (i) follicles need to be detached from the ovaries for ovulation and GVBD to occur and (ii) within 30–40 min after follicular detachment from the ovaries, oocyte maturation is triggered by a maturation-inducing substance that is transferred from the follicle cells to the oocyte via junctional complexes connecting follicle cells to the oolemma. The results of this investigation are compared to previously published data on brachiopods, and the events observed during *in vitro* maturation are discussed with respect to how oocyte maturation may be accomplished during normal development in the field.

Materials and Methods

Collection of animals and handling of gametes

Adult specimens of *Terebratalia transversa* (Sowerby, 1846) measuring 20–40 mm in shell width were collected by dredging or scuba diving in the vicinity of San Juan Island, Washington. Most females obtained during the summer had incompletely developed oocytes that did not successfully undergo maturation or fertilization, whereas fully gravid specimens occurred more commonly during the peak spawning season in the winter. Thus, to enhance the numbers of ripe specimens for studies of oocyte maturation, intact females were stored for several months in laboratory aquaria with running seawater so that, as pre-

viously noted by Reed (1987), developmentally competent oocytes could accumulate in the ovaries.

Observations and experiments were conducted during May to August, 1995 and 1996, or from December 1996 to February 1997. Specimens used in this study had moderately to fully ripe ovaries, and at least 50% levels of ovulation and GVBD were attained after oocyte maturation was triggered *in vitro*. In cases where ovaries contained mixed stages of oogenesis, relatively small oocytes that were incapable of undergoing maturation were ignored; the dataset thus included only well-developed specimens that measured $>100\ \mu\text{m}$ in diameter and occurred within a prominent follicular sheath situated at least $5\ \mu\text{m}$ from the surface of the oocyte; *i.e.*, those essentially corresponding to “stage IV oocytes” in the four-stage classification scheme of Long (1964).

For analyses of oocyte maturation, the bivalved shell of each adult female was pried apart with a scalpel, and the inner surfaces of the shell valves were scraped with a glass pipette to dislodge follicles from the ovarian tissue that lay near the shell. Follicles that were isolated from ripe ovaries by such maceration techniques were rapidly washed once with filtered seawater (SW) and subsequently kept at 12° – 16°C .

Microscopy

For light microscopic observations, isolated follicles that had been obtained from macerated ovaries were placed in specimen dishes maintained at 12° – 16°C by a thermoelectric cooling stage (Stricker, 1994). Oocyte maturation was then monitored by video microscopy, using an inverted microscope, CCD (charge-coupled device) video camera, and a time-lapse video recorder set at 70–120 $\times$ time-compression (Stricker *et al.*, 1994a). Alternatively, maturation was also examined by means of either a Nikon Optiphot photomicroscope equipped with 35-mm camera or a Bio-Rad MRC-600 confocal microscope employing nonconfocal, transmitted-light optics (Stricker *et al.*, 1994b).

To verify the patterns observed in living specimens, cultures of isolated follicles were incubated at 12° – 16°C and serially fixed in formalin/glutaraldehyde (Stricker, 1995). Some of the fixed specimens were subsequently treated with a Triton- and glycerol-containing solution to render the oocytes more translucent (Stricker and Schatten, 1989), whereas nuclear architectural changes were monitored in follicles that had been labeled with the DNA-binding dye Hoechst 33342 (Sigma) prior to fixation (Stricker, 1995).

For transmission electron microscopy (TEM), pieces of ovaries or isolated oocytes were doubly fixed in glutaraldehyde and osmium tetroxide, dehydrated in ethanol, and embedded in “LX-112” Epon equivalent (Ladd Research Co.) (Stricker *et al.*, 1992a). After $1\text{-}\mu\text{m}$ sections

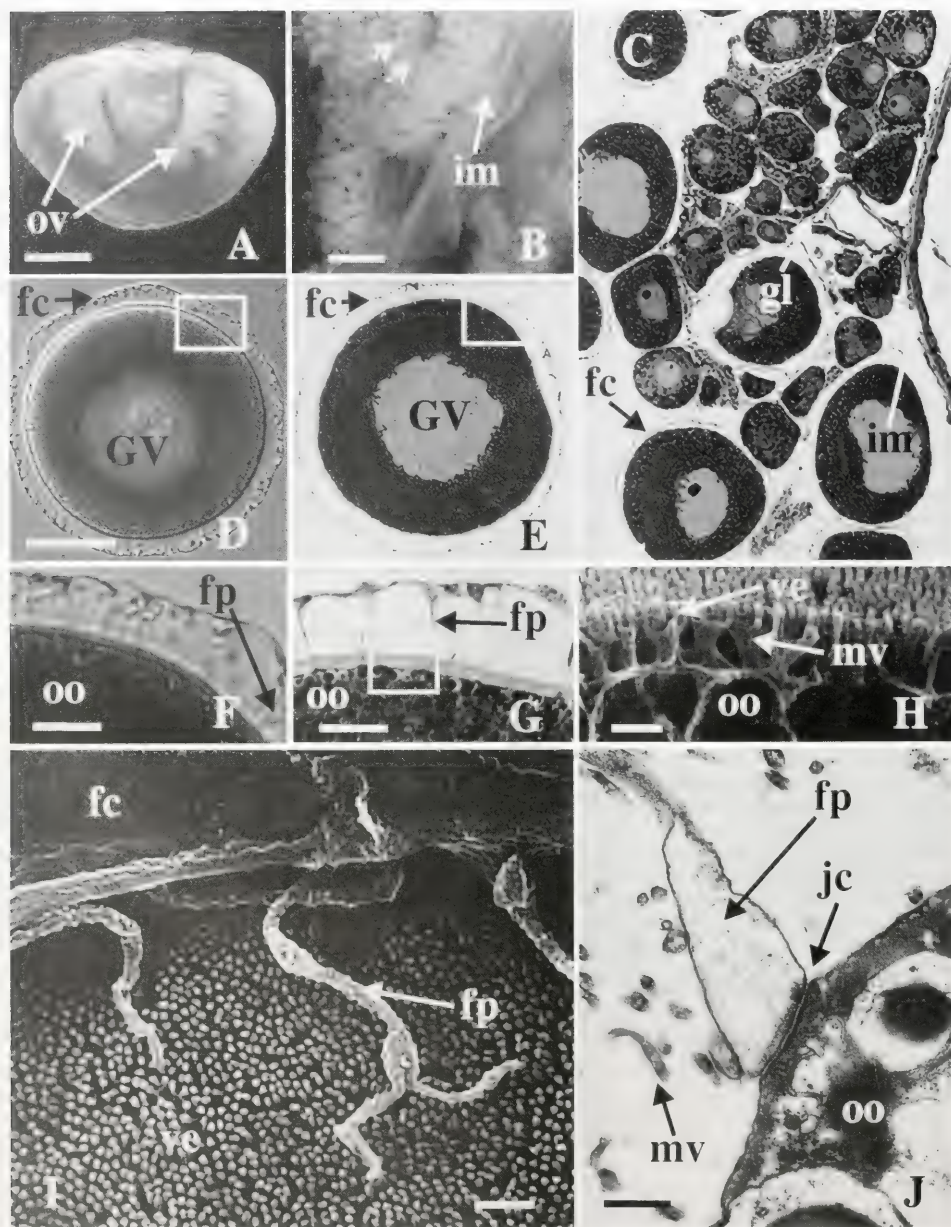


Figure 1. (A) Video macrograph of the dorsal valve of a ripe *Terebratalia transversa* female with the lophophore and viscera removed to show the U-shaped ovarian lobes (ov) that lie between the inner and outer mantle membranes underlying the shell; scale bar = 5 mm. (B) Video macrograph, showing that after

were obtained for orientation, the embedded specimens were sectioned at 70 nm and stained with uranyl acetate followed by lead citrate (Stricker *et al.*, 1992a). For scanning electron microscopy (SEM), isolated follicles or follicle-cell-free oocytes were fixed in a bicarbonate-buffered solution of osmium tetroxide, dehydrated in ethanol, dried by the critical-point method, and coated with gold-palladium (Stricker and Cloney, 1983).

Video images were digitized using an Image-1 image processing system (Universal Imaging Corp.), whereas EM or 35-mm negatives were printed, and the prints were subsequently digitized at 500 dpi using a UMAX S8 flat-bed scanner. Some of the digital files were subjected to an "unsharp mask" or a "sharpening" kernel to enhance details before being constructed into montages using the MetaMorph software of Universal Imaging Corp. The montages were then labeled with CorelDraw 7.0 and recorded on 4X5 T-Max 100 film using an Agfa QZR-C film recorder (Stricker *et al.*, 1994b).

Experimental manipulations

To test the effects of mechanically removing follicle cells, a piece of Nitex (85–95 μ m mesh) was glued over the cut front end of a 5-ml syringe to construct a cylindrical filter. At various time-points after maceration of the ovaries, the isolated follicles were drawn through the Nitex-covered syringe 5–10 times so that nearly all of the oocytes were completely stripped of their follicle cells without significantly damaging the oocytes.

For analyses of microfilament-mediated processes, isolated follicles were incubated in a SW solution of the actin-depolymerizing agent cytochalasin D (CD) (Sigma) that was prepared from a 10 mM stock mixture in dimethylsulfoxide (DMSO) (Sigma). One drop (~50 μ l) of follicles was added to 1.5 ml of 10 μ M CD, based on previous findings that 10 μ M solutions of cytochalasin inhibited ovulation and

GVBD in starfish oocytes (Schroeder, 1971; Stricker and Schatten, 1991). Controls received 0.1% DMSO without CD.

To determine the effects of altering the adhesion of follicle cells to oocytes, isolated follicles were incubated in calcium-free seawater (CaFSW) (Schroeder and Stricker, 1983) or a 1% seawater solution of trypsin (Sigma). For the CaFSW treatments, follicles were gently washed with numerous changes of CaFSW to ensure that external calcium concentrations were negligible.

Alternatively, seawater solutions of drugs that uncouple the gap junctions formed by various cell types (Davidson *et al.*, 1986; Sandberg *et al.*, 1990; Downs, 1995; Germain and Ancil, 1996) were applied to isolated follicles to test whether intercellular communication via such junctions is required for normal maturation. The drugs were purchased from Sigma and consisted of 1 mM heptanol or 25 μ M 18- α -glycyrrhetic acid (AGA), based on the effective levels used on other oocytes (Patino and Purkiss, 1993; Downs, 1995). The AGA solutions were prepared from a 5 mM stock made in DMSO, and 0.5% DMSO was added to the control treatments.

For experimental analyses, oocytes were collected from at least two females, and the experiments were typically repeated three or more times. To assess the percentages of maturation and the sizes of oocytes, 60–200 oocytes were examined per ovary or timepoint of data collection. Statistical analyses involved Student's *t* tests, or Mann-Whitney *U* tests for large and small datasets, respectively (Sokal and Rohlf, 1973). Sample sizes for the timing of maturation processes varied, since not all events could be clearly discerned in each specimen.

Results

Morphology of the ovaries and prophase-arrested oocytes

In gravid females, a bilobed, U-shaped ovary occurred between the inner and outer mantle membranes underly-

the inner mantle membrane (im) is reflected with fine forceps to expose a ripe ovary, the follicles (arrows) remain attached to the genital lamellae of the ovary; scale bar = 500 μ m. (C) Epon section (1 μ m) of an incompletely ripe ovary, showing oocytes at various stages of oogenesis attached to the genital lamellae (gl) that arise from the inner mantle (im); well-developed oocytes are surrounded by a sheath of follicle cells (fc); scale bar = 50 μ m. (D) Photomicrograph of an isolated follicle obtained after macerating a ripe ovary; fc = follicle cells; GV = germinal vesicle; the rectangle outlines a region similar to that shown at higher magnification in 1F; scale bar = 50 μ m. (E) Epon section (1 μ m) of an isolated follicle, showing the surrounding follicle cells (fc) and the centrally located germinal vesicle (GV); the rectangle outlines a region similar to that shown at higher magnification in 1G; scale bar = 50 μ m. (F) Transmitted-light image, obtained using a confocal microscope, of follicle-cell projections (fp) that attach to the oolemma of fully developed oocytes (oo); scale bar = 10 μ m. (G) Epon section (1 μ m) of the follicle-cell projections (fp) that attach to the surface of the oocyte (oo); the rectangle outlines a region similar to that shown at higher magnification in 1H; scale bar = 10 μ m. (H) Scanning electron micrograph (SEM) of a fractured oocyte (oo) that is no longer surrounded by a follicular sheath. The vitelline envelope (ve) comprises an interconnected meshwork of filaments at the apices of the oocyte's microvilli (mv); scale bar = 1 μ m. (I) SEM of the follicle cell projections (fp) that penetrate the vitelline envelope (ve) to attach to the oocyte via junctional complexes; fc = follicle cells; scale bar = 1 μ m. (J) Transmission electron micrograph of a follicle cell projection (fp) and the junctional complex (jc) it makes with the oolemma of the oocyte (oo); mv = microvilli; scale bar = 0.5 μ m.

ing the dorsal and ventral valve of each shell (Fig. 1A, B). Such ovarian lobes consisted of a series of folds, or "genital lamellae" (James *et al.*, 1991a), that were covered throughout by a germinal epithelium, to which clusters of developing oocytes and fully formed follicles were connected (Fig. 1C). After the inner mantle was gently reflected with fine forceps to uncover a ripe ovary, the exposed gonad routinely maintained an intact outline (Fig. 1B), suggesting that the follicles remained attached to the germinal epithelium and were not freely floating in the space between the inner and outer mantle.

Before the onset of oocyte maturation, each follicle comprised a prophase-arrested oocyte and its enveloping sheath of follicle cells. The diameter of fully grown oocytes averaged $131 \pm 10 \mu\text{m}$ in 15 specimens examined, and the size of such oocytes did not differ markedly in ventral *versus* dorsal ovaries or in winter *versus* summer. Each well-differentiated oocyte contained a centrally located GV that had a single large nucleolus and involuted nuclear envelope (Fig. 1D, E). Many branched microvilli arose from the surface of the oocyte and were interconnected at their apices by a meshwork of crosslinked filaments that collectively constituted an extremely hardy vitelline envelope (Fig. 1F-H).

The follicular sheath surrounding each prophase-arrested oocyte consisted of roughly two dozen flattened, nonciliated cells with tightly joined boundaries. In fully differentiated follicles corresponding to stage IV of Long (1964), the follicle cells were situated about $10 \mu\text{m}$ from the surface of the oocyte and extended many slender projections toward the oolemma (Fig. 1D-G). These follicle-cell projections penetrated the vitelline envelope (Fig. 1I) and connected with the oolemma by means of junctional complexes (Fig. 1J) that resembled the junctions reported for other marine invertebrate follicles (Anderson, 1969; Schroeder *et al.*, 1979; Schroeder, 1981).

Triggers of *in vitro* maturation

As noted previously (Long, 1964; Reed, 1987; Freeman, 1993), *in vitro* maturation can be triggered simply by macerating ripe ovaries of *T. transversa*. Fully formed follicles that were isolated by this method completed ovulation and GVBD in tandem, with ovulated oocytes undergoing GVBD, and GVBD failing to occur in oocytes that did not ovulate. In five ovaries containing relatively small oocytes (avg. diameter = $114 \pm 8 \mu\text{m}$), ovulation and GVBD occurred in less than 5% of the isolated follicles examined. Such nonmaturing oocytes did not develop upon insemination, whereas relatively high percentages (>80%) of normal development were achieved if oocytes were fertilized following GVBD (pers. obs.).

To assess which parameters of the maceration procedure were necessary for triggering oocyte maturation, a few concentrated drops of isolated follicles were placed

in 10 ml of filtered SW within 30 s of ovarian maceration, and the follicles were rapidly washed again with 10 ml of SW. In all nine trials, washed oocytes continued to mature at the same levels as observed in follicles maintained in the macerated ovarian fluids (pers. obs.). This result indicates that any soluble substances released by the maceration procedure were not continuously required for maturation or were not diluted to subthreshold levels by the double washes in seawater. Conversely, to determine maturation levels in nonmacerated ovaries exposed to seawater, isolated shell valves with intact ovaries were immersed in seawater, whereas the ovarian lobes from the other half of each animal were macerated to trigger oocyte maturation. After being incubated for 3 h, only $9\% \pm 8\%$ of the oocytes aspirated with a pipette from intact ovaries ($n = 7$) had matured, but a significantly higher percentage ($92\% \pm 10\%$; $n = 7$; $P < 0.05$) of the follicles isolated from macerated ovaries had matured. This result indicates that the simple exposure of follicles to seawater cannot fully account for the high levels of *in vitro* maturation that are achieved by disrupting ovarian tissues.

To test for possible maturation-inducing substances released by the ovaries during maceration, shell valves with intact ovaries were placed upright in bowls and treated with 1–2 ml of either filtered SW or an "ovarian supernatant" that was freshly obtained by macerating the ovary from the other half of the animal in 1–2 ml of seawater, and then centrifuging the macerated tissues and fluids at $14,000 \times g$ for 1 min to produce a cell-free supernatant. These solutions were either injected beneath the inner mantle with a 25-gauge syringe, or the valves were cut in half with a scalpel so that the ovarian lobe on each half-valve could be immersed in the test solution. After 3 h of incubation, follicles were aspirated from the ovaries with a pipette, and while doing so, care was taken to avoid any oocytes that had become dislodged during handling of the ovaries. As is evident in Table I, treatments with either seawater or ovarian supernatant failed to trigger significant levels of maturation in intact ovaries by 3 h post-treatment. The slightly elevated maturation percentages compared to the $9\% \pm 8\%$ average obtained in previous seawater treatments of intact ovaries could be attributed simply to the presence of more detached oocytes (dislodged by the injections or valve bisections) than in seawater incubations of intact valves.

In other experiments involving more prolonged incubations, the ovarian tissue on one shell valve was macerated to provide both an ovarian supernatant and a set of control follicles that were detached from the ovaries. The other shell valve was then bisected with a scalpel so that one ovarian lobe was immersed in the ovarian supernatant, while the other intact lobe was covered with seawater. After 20 h of incubation, the number of mature oocytes in the intact ovaries treated with the supernatant more

Table I

Percentages of mature oocytes in intact ovaries vs. detached follicles of *Terebratalia transversa*

Duration of treatment (h)	n ³	Intact ovaries ¹		Detached follicles ²
		Seawater	Ovarian supernatant	Controls
3	5	19 ± 29%	23 ± 35%	91 ± 7% [†]
20	3	17 ± 12%	59 ± 27% ^{**}	96 ± 6% ^{**}

¹ For experimental treatments, an intact ovarian lobe on a bisected shell valve was immersed in either seawater or a supernatant obtained from macerated ovarian tissues. Alternatively, a single whole shell valve with intact ovarian lobes was given a submantle injection of either seawater or the ovarian supernatant. After 3 or 20 h of treatment, oocytes were aspirated from the ovaries, and the percentage of maturation was assessed.

² Controls consisted of detached follicles that were obtained from the macerated ovarian tissues used for the production of the ovarian supernatant. Maturation rates were assessed in these isolated follicles 3 or 20 h after ovarian maceration.

³ Number of specimens in each treatment or control.

[†] Significantly greater than the seawater or supernatant treatments ($P < 0.05$).

^{**} Significantly greater than the seawater treatment ($P < 0.05$).

closely approached the values obtained in the macerated controls (Table I). Conversely, the percentage of maturation in intact ovaries treated with seawater was not as high as in the supernatant trials (Table I).

Timing and patterns of ovulation and germinal vesicle breakdown

After detachment from the ovary, the follicular sheath of more than 90% of the fully grown follicles examined underwent a dynamic retraction to form a cap of aggregated follicle cells at one pole of the oocyte (Fig. 2A–H). In individual specimens monitored by time-lapse video microscopy at 12°–16°C, cap formation occurred 81 ± 15 min ($n = 71$) after detachment of the follicles from the ovaries (Fig. 3). This timing was also corroborated by serial fixations of mass cultures that interpolated the time at which 50% of the culture had undergone follicle-cell capping (= " $T_{1/2}$ ovulation") as 77 ± 20 min ($n = 7$).

Follicle-cell projections started to withdraw from the oolemma 19 ± 9 min ($n = 47$) before the cap was formed, and follicle-cell capping was accomplished in 8 ± 3 min ($n = 30$) from the onset of a poleward movement to the formation of a distinct cap. After ovulation was completed, the exposed vitelline envelope contained numerous holes that presumably represented the former sites of follicle-cell penetrations through the envelope, and the caps eventually became dislodged from the oocytes (Fig. 2I). Whether a preferred site of cap formation existed relative to the animal-vegetal axis of the oocyte was not determined, because

the GV tended to be centrally located in the oocyte and follicle-cell caps were not present at polar body formation to provide landmarks for orientation.

In six batches of oocytes fixed 3 h after detachment of the follicles from the ovaries, GVBD occurred in 92% ± 9% of the isolated follicles. Based on time-lapse video analyses of living specimens, GVBD began with the disassembly of the nuclear envelope 8 ± 3 min ($n = 13$) after the first noticeable withdrawal of follicle-cell projections from the oolemma. Accordingly, incompletely ovulated oocytes contained a GV with indistinct boundaries (Fig. 2J), and in three fixation series, the time at which 50% of the specimens had already begun GVBD (" $T_{1/2}$ GVBD onset") averaged 70 ± 12 min after removal of the follicles from the ovaries versus a $T_{1/2}$ ovulation time of 80 ± 9 min post-removal.

After nuclear envelope disassembly, the nucleoplasm mixed with the surrounding cytoplasm, and the nucleolus eventually disintegrated to mark the end of GVBD (Fig. 2K, L), which in turn occurred 24 ± 16 min after the completion of ovulation ($n = 6$). By 3 h after removal of the follicle from the ovary, oocytes that had originally contained a GV and distinct nucleolus (Fig. 4A) possessed a circular array of chromosomes at the metaphase plate (Fig. 4B). Following insemination, such post-GVBD oocytes routinely cleaved and developed into normal larvae (Reed, 1987; pers. obs.).

Experimental manipulations of ovulation

To determine the effects of inhibiting ovulation, washed follicles were immersed in SW solutions of cytochalasin D within 5 min after detachment of the follicles from the ovaries. Following 3 h of incubation, 10 μ M CD blocked follicle-cell capping in more than 90% of the oocytes, whereas 1 μ M CD inhibited ovulation in less than 5% of the follicles (pers. obs.; Fig. 4C–G). This inhibition was reversed by washing CD-treated follicles in SW (Figs. 4F, G; 5), and ovulation was verified in time-lapse video studies of the washed specimens (pers. obs.), suggesting that rather than killing follicle cells, the CD treatment temporarily inhibited follicle-cell capping by disrupting microfilament-mediated movements; such disruption has been noted for other marine invertebrates (Schroeder, 1971; Smiley and Cloney, 1985).

After about 1 h in the CD solution, the flattened follicle cells rounded up, became separated by large gaps in what was previously a contiguous follicular sheath, and acquired spindly projections with knoblike endings (Fig. 4C–E). However, GVBD still proceeded in these morphologically altered follicles, as was apparent from images of intact specimens and sectioned material (Fig. 4C, D). In comparison to the more than 90% ovulation and GVBD obtained for control specimens not receiving CD, an average of 79% ± 9% of the CD-treated specimens

had undergone GVBD by 3 h post-incubation, but only 8% $\pm$ 17% of these oocytes were devoid of follicle cells in 17 trials using 10 μ M CD. Thus, the CD treatments significantly ($P < 0.05$) reduced the percentage of ovulation, but not that of GVBD.

In contrast, isolated follicles that were treated with CaFSW to accelerate, rather than to inhibit, ovulation routinely retained their full complement of follicle cells directly after washing, and they subsequently completed ovulation and GVBD (Figs. 4H). However, as seen in four sets of serial fixations, CaFSW significantly accelerated the rate of ovulation ($P < 0.05$) and slightly decreased the time before the onset of GVBD, compared to control cohorts that were incubated in SW (CaFSW: $T_{1/2}$ ovulation = 60 $\pm$ 19 min; $T_{1/2}$ GVBD onset = 65 $\pm$ 18 min vs. SW: $T_{1/2}$ ovulation = 79 $\pm$ 21 min; $T_{1/2}$ GVBD onset = 69 $\pm$ 17 min) (Fig. 6). Treatments with 1% seawater solutions of trypsin, which caused follicle cells to lie close to the oolemma (Fig. 4I), also altered maturation kinetics as observed following CaFSW treatments (Trypsin: $T_{1/2}$ ovulation = 61 $\pm$ 3 min; $T_{1/2}$ GVBD onset = 60 $\pm$ 11 min vs. SW: $T_{1/2}$ ovulation = 80 $\pm$ 13 min; $T_{1/2}$ GVBD onset = 73 $\pm$ 11 min). After being washed in SW and subsequently inseminated, such CaFSW- or trypsin-treated specimens developed into larvae (Fig. 4J) resembling those that could be successfully triggered to metamorphose (Stricker and Reed, 1985), collectively indicating that ovulation could be accelerated without preventing normal development.

Similarly, for undetermined reasons, oocytes obtained from ventral ovaries completed ovulation more rapidly than did cohort oocytes collected from dorsal ovaries (ventral ovaries: $T_{1/2}$ ovulation = 73 $\pm$ 11 min; dorsal ovaries: 98 $\pm$ 19 min; $n = 5$; $P < 0.05$). Moreover, by macerating only one ovarian lobe on each half of the shell and then allowing the other lobe to be pre-incubated in seawater for at least 3 h before being macerated, the rates

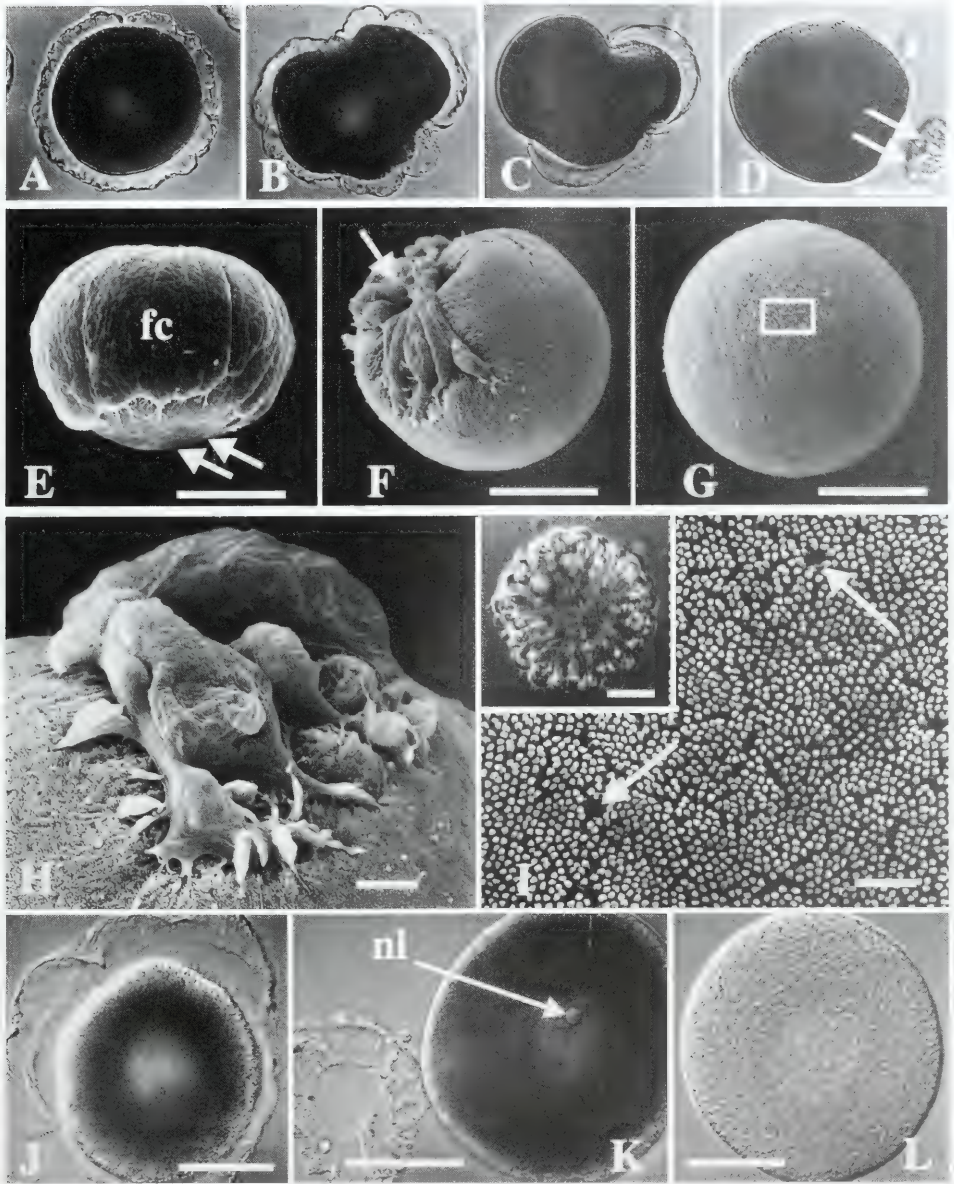
of ovulation were significantly faster in the pre-incubated specimens than in the non-incubated controls (pre-incubated ovaries: $T_{1/2}$ ovulation = 59 $\pm$ 14 min; non-incubated ovaries: 84 $\pm$ 12 min; $n = 8$; $P < 0.05$).

Effects of altering follicle cell–oocyte attachments during oocyte maturation

To determine whether follicle cells need to be attached to the oocyte for GVBD to occur, a Nitex filter was used to separate oocytes from their surrounding follicular sheaths at various timepoints following ovarian maceration (Fig. 7A). If follicle cells were stripped up to 30 min after detachment of the follicles from the ovaries, very few of the follicle-cell-free oocytes completed GVBD (Figs. 7B, 8), and the block was essentially permanent, because in all eight cultures that had been stripped of follicle cells at 5 min post-removal from the ovary, the levels of GVBD remained lower than 10% when examined the next morning. Such precocious removals of follicle cells inhibited GVBD whether the stripped oocytes were kept in the same solution with the isolated follicle cells or were placed in fresh SW lacking the follicle cells. Moreover, simply transferring control specimens with intact follicular sheaths to another SW solution 15 min after ovarian maceration did not prevent ovulation or GVBD. These results collectively indicate that the inhibition of GVBD was not due simply to the dilution of soluble cues generated by the maceration procedure.

In contrast, specimens stripped of their surrounding follicle sheath more than 40 min after removal of the follicles from the ovaries consistently showed high levels of GVBD (Figs. 7C, 8), which suggests that the stripping procedure did not block GVBD by merely increasing cell morbidity. The time at which follicle cell removal was accompanied by only a 50% level of GVBD (= the " $T_{1/2}$ inhibitory time") averaged 37 $\pm$ 12 min ($n = 16$ ovaries).

Figure 2. (A–D) Photomicrographs taken over a period of about 90 min during the ovulation of a single living oocyte of *Terebratalia transversa* that was obtained after macerating a ripe ovary. The sequence shows the retraction of the follicular sheath to form a cap of follicle cells that eventually dislodges from the ovulated oocyte (arrows in 2D); scale bar for A–D = 50 μ m. (E) SEM of an isolated follicle that was fixed less than 5 min after maceration of the ovary; fc = follicle cells; the arrows mark the exposed end of the oocyte that was previously attached to the germinal epithelium within the ovary prior to ovarian maceration; scale bar = 50 μ m. (F) SEM of a follicle that was fixed about 75 min after removal of the ovary, showing a nearly completed follicle cap (arrow) at one pole of the ovulating oocyte; scale bar = 50 μ m. (G) SEM of a completely ovulated oocyte, fixed 3 h after removing the follicle from the ovary; the rectangle outlines a region similar to that shown at higher magnification in 2I; scale bar = 50 μ m. (H) SEM of the cap of follicle cells (arrows) that forms at one pole of the oocyte during ovulation; scale bar = 10 μ m. (I) SEM of the surface of an ovulated oocyte fixed 3 h after removing the follicle from the ovary, showing holes (arrows) in the vitelline envelope through which follicle-cell projections presumably extended prior to ovulation; scale bar = 1 μ m; inset: photomicrograph of a cap of follicle cells that had become detached from the oocyte following ovulation; scale bar = 10 μ m. (J) Photomicrograph of an incompletely ovulated oocyte with a follicle cell sheath that was in the process of being retracted at the time of fixation (about 70 min after removal of the follicle from the ovary); germinal vesicle breakdown (GVBD) had begun before ovulation was completed, as evidenced by the irregular outline of the GV; scale bar = 50 μ m.



(K) Photomicrograph of an oocyte fixed about 75 min after removing the follicle from the ovary; the partially retracted sheath of follicle cells was dislodged from the oocyte surface during preparation of the specimen for microscopy; note that GVBD had begun, but the nucleolus (nl) is still present; scale bar = 50 μ m. (L) Photomicrograph of an oocyte fixed 2 h after removal of the follicle from the ovary; both ovulation and GVBD have been completed; scale bar = 50 μ m.

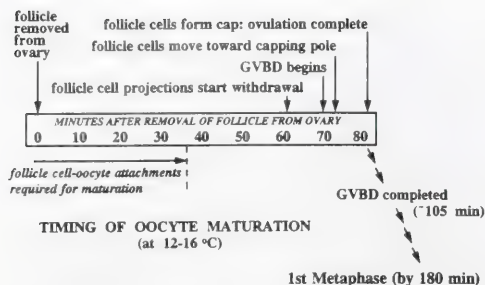


Figure 3. Summary of the timecourse of events during normal oocyte maturation.

In comparison, the $T_{1/2}$ ovulation time for cohort controls not stripped of their follicular sheath was 79 ± 18 min post-detachment of the follicles from the ovaries ($n = 16$), and the control groups averaged $>90\%$ GVBD by 3 h after ovarian maceration.

To determine whether gap-junction uncouplers can affect the percentage of oocyte maturation achieved, intact ovaries were immersed for 1–2 h in $12^\circ\text{--}16^\circ\text{C}$ SW solutions of 1 mM heptanol or $25 \mu\text{M}$ α -glycyrrhetic acid (AGA) before being macerated. This method was chosen because pre-incubations of 30 min have been used to uncouple mammalian follicle cell–oocyte gap junctions at 37°C (Downs, 1995). After the pre-incubated ovaries were macerated, isolated follicles were soaked in the gap junction uncoupler for another 3 h. Both gap-junction uncouplers significantly reduced ($P < 0.05$) the numbers of oocytes that fully matured (*i.e.*, ovulated and underwent GVBD) (Fig. 7D, E). Moreover, the decrease was not solely due to cell morbidity, because heptanol- or AGA-treated samples that had been washed overnight in seawater had higher percentages of maturation the next morning and were able to produce normal blastulae after fertilization (*pers. obs.*).

Furthermore, many drug-treated oocytes underwent GVBD but failed to complete ovulation (Fig. 7F, G). Thus, compared to the $93\% \pm 10\%$ level of ovulation and accompanying GVBD in six batches of controls not receiving the gap-junction uncouplers, heptanol treatments reduced GVBD to $40\% \pm 40\%$ and decreased the level of full ovulation to $32\% \pm 39\%$ ($n = 6$). Similarly, applications of the AGA uncoupler yielded $66\% \pm 33\%$ and $33\% \pm 22\%$ levels of GVBD and successful ovulation, respectively ($n = 6$), which in turn suggests that the

drug treatment may have also affected the transmission of signals that enable ovulation to proceed normally.

To assess the effects of such uncoupling drugs on GVBD without incubation of the intact ovaries, isolated follicles were removed from macerated ovaries and transferred directly to 1 mM heptanol or $25 \mu\text{M}$ AGA. As shown in Figure 9, the percentages of GVBD in the unincubated, heptanol-treated specimens were significantly higher ($P < 0.05$; $n = 6$) than those attained following a 1–2 h pre-incubation in heptanol, and unincubated AGA-treated follicles exceeded the levels of GVBD displayed by controls. Pre-incubation may thus be needed to achieve optimal gap-junction uncoupling before maturation is triggered.

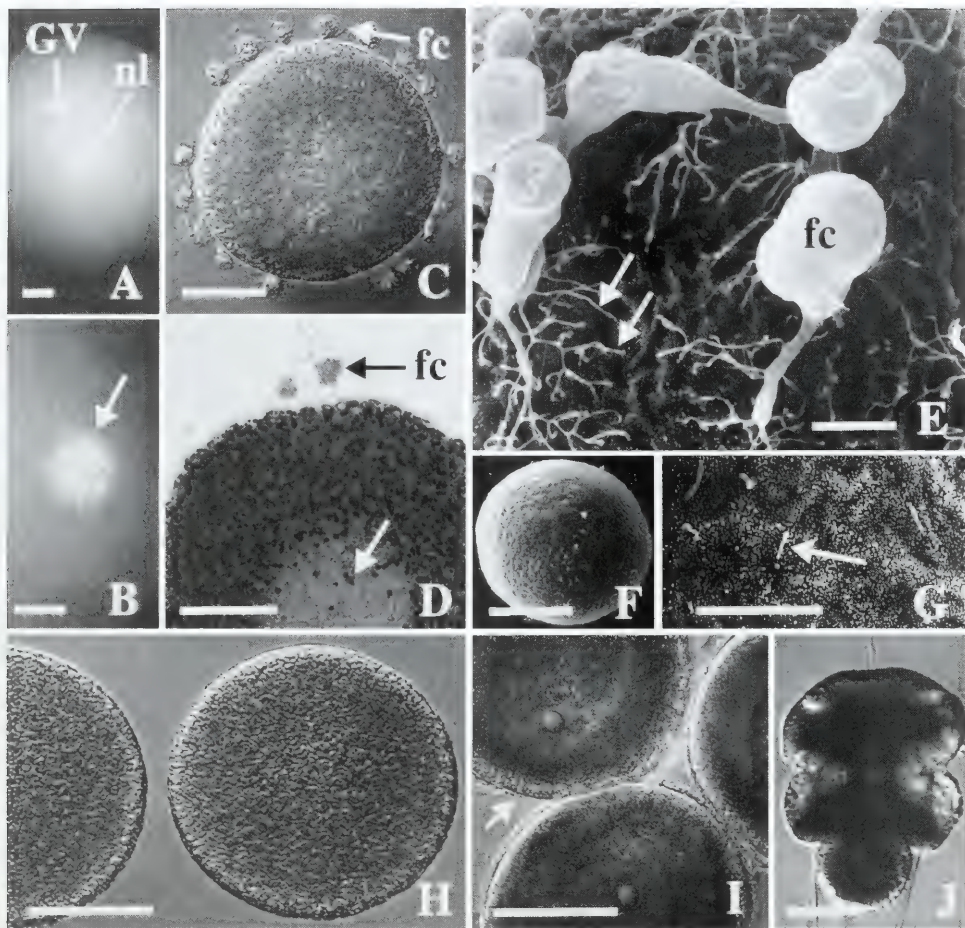
In view of these results on the effect of pre-incubation and the observation that follicles undergo morphological alterations an hour after being treated with CD, intact ovarian lobes on bisected shell valves were placed in a $10\text{-}\mu\text{M}$ SW solution of CD for 1–2 h before the pre-incubated ovaries were macerated, and the isolated follicles were immersed for another 3 h in CD (Figs. 7H, 10). After the additional 3 h in the CD solution, the percentage of GVBD was reduced to $33\% \pm 33\%$ ($n = 7$). This maturation level was significantly lower ($P < 0.05$) than the $81\% \pm 30\%$ ($n = 7$) GVBD level obtained in CD-treated oocytes from cohort ovaries ($n = 7$) that were not pre-incubated in CD but only immersed in it for 3 h after ovarian maceration, which in turn suggests that GVBD is inhibited by a prior incubation in CD that allows follicle cell–oocyte attachments enough time to be altered before oocyte maturation can be triggered.

Discussion

In vitro maturation of T. transversa oocytes in relation to detachment of follicles from the ovaries

To provide a positive stimulus for oocyte maturation, the follicle cells of many animals generate maturation-inducing factors in response to a gonad-stimulating substance (GSS) supplied by extraovarian tissues. For example, the GSS of starfish is produced in the vicinity of the radial nerves and transported to the gonads where it can cause follicle cells to secrete the maturation inducer 1-methyladenine (Meijer and Guerrier, 1984). Similarly, the gonadotropic hormones of vertebrates are gonad-stimulating substances secreted by extraovarian tissues; these hormones can cause the release of maturation-inducing steroids from follicle cells (Masui and Clarke, 1979). Alternatively, mammalian oocytes can be triggered to undergo

Figure 4. (A) Photomicrograph of a Hoechst-labeled oocyte 10 min after removal from the ovary, showing an intact germinal vesicle (GV) with a brightly fluorescent nucleolus (nl); note: the follicle cells had been removed during processing of the specimen; scale bar = $10 \mu\text{m}$. (B) Photomicrograph of a Hoechst-



labeled oocyte 3 h after removal from the ovary, showing a circular set of chromosomes (arrow) at the metaphase plate; scale bar = 10 μ m. (C) Photomicrograph taken 3 h after an oocyte was removed from the ovary and directly transferred into 10 μ M cytochalasin D to block follicle cell (fc) capping. Such CD-treated oocytes nevertheless undergo GVBD; scale bar = 50 μ m. (D) Epon section (1 μ m) of a CD-treated oocyte at 3 h post-treatment, showing follicle cells (fc) still surrounding the oocyte, but the absence of an intact nuclear envelope, as evidenced by the mixing of nucleoplasm with cytoplasmic granules (arrow); scale bar = 50 μ m. (E) SEM of a CD-treated oocyte at 3 h post-treatment, showing follicle cells (fc), which round up and acquire spindly projections with knoblike endings (arrows) starting around 1 h post-treatment; scale bar = 10 μ m. (F) SEM of a CD-treated oocyte that had been subsequently washed in SW to allow the shedding of follicle cells; scale bar = 50 μ m. (G) SEM of the surface of a CD-treated oocyte that had been subsequently washed in SW to allow the shedding of follicle cells; a few follicle cell projections (arrow) remain; scale bar = 10 μ m. (H) Photomicrograph of two oocytes that had completed ovulation and GVBD within 2.5 h after treatment with calcium-free seawater; scale bar = 50 μ m. (I) Photomicrograph of three follicles that had been treated with a 1% seawater trypsin solution for 30 min after removal from the ovary; the follicle cells (arrow) of such trypsin-treated specimens lay close to the oolemma, but eventually undergo retraction, coupled with GVBD; scale bar = 50 μ m. (J) Photomicrograph of an approximately 3-day-old larva that developed from a CaFSW-treated follicle, which was subsequently washed in seawater and inseminated after GVBD; scale bar = 50 μ m.

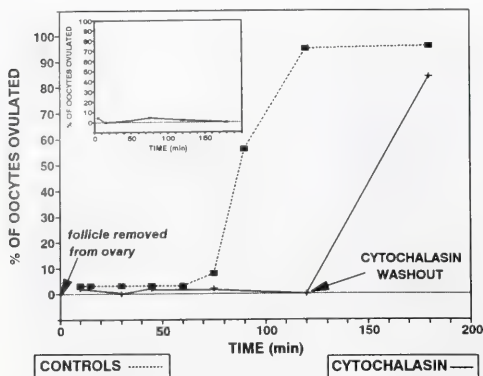


Figure 5. Follicles isolated from a macerated ovary and subsequently incubated in $10 \mu\text{M}$ cytochalasin D fail to undergo ovulation by 3 h post-treatment (inset). However, if the cytochalasin is washed out at 2 h post-treatment, essentially the same percentage of ovulation occurs in follicles previously treated with cytochalasin as in control specimens that were obtained from the same ovary but were not subjected to cytochalasin. Such findings indicate that the cytochalasin treatment only temporarily blocks ovulation and does not prevent follicle-cell capping simply by killing the follicles.

spontaneous maturation by removing their follicular sheaths, thus eliminating the inhibitory "arrestor" signal provided by the follicle cells (Cho *et al.*, 1974; Racowsky and Baldwin, 1989; Downs, 1997).

Results from this analysis of *T. transversa* fail to provide evidence either that an inhibitory substance is supplied by the follicle cells or that an extraovarian GSS is required during *in vitro* maturation. The lack of follicular inhibition of maturation is demonstrated by the failure of *T. transversa* oocytes to mature if they are mechanically stripped of their follicle cells within 30 min of being isolated from the ovaries. Similarly, gap-junction uncouplers, which would presumably prevent inhibitors from passing through the junctions connecting follicle cells to the oocyte, cause a drop in maturation rather than the increase that would be expected if maturation were arrested simply as a result of the inhibition provided by an intact follicular sheath.

Oocyte maturation in the apparent absence of an extraovarian GSS is suggested by the finding that oocytes regularly mature following maceration of just the dorsal or ventral ovaries. Thus, if a GSS is required for *in vitro* maturation, it either is produced in the ovaries or adjacent tissues, or it is somehow stored in these regions after being generated at other extraovarian sites. The requirement for a GSS is further questionable considering that oocytes continue to mature after the fluids generated by ovarian macerations are rapidly replaced with SW. Hence, if a soluble GSS is required, it either affects follicle cells

almost instantaneously upon release or is active at concentrations lower than those obtained by the washing procedure used in this study. Moreover, a supernatant derived from macerated ovaries does not immediately promote high levels of maturation in intact ovaries, indicating that if a soluble GSS is indeed released by maceration, the procedure used in this study failed to extract it at a concentration capable of eliciting maturation of intraovarian follicles within 3 h after treatment.

Alternatively, *in vitro* maturation may simply depend on the physical detachment of the follicle from the ovary (Fig. 11). This possibility is consistent with the observation that ripe ovaries examined in this study seemed to lack free-floating follicles. Accordingly, reports of *T. transversa* follicles being detached from the ovarian wall (Long, 1964) might reflect follicles dislodged during specimen handling. In fully ripe females, dislodging can occur if the shell valves are not opened gently enough (pers. obs.). Judging from scanning electron micrographs of freshly isolated follicles, attached intraovarian follicles presumably possess a complete follicular sheath that shields the enclosed oocyte from external cues such as injected seawater. Moreover, immediately after removal from the ovaries, virtually all oocytes have an intact GV. Collectively, these observations suggest that an intraovarian oocyte remains arrested at prophase I as long as it is fully encased by a follicular sheath that is connected to the germinal epithelium.

Following mechanical disruption of the ovary, each detached follicle acquires an opening at one end of the follicular sheath, exposing the oocyte to seawater. In some other animals, such exposure may trigger GVBD (Stricker, 1996), presumably by altering the levels of pH, calcium, or other ions from those maintained in intact,

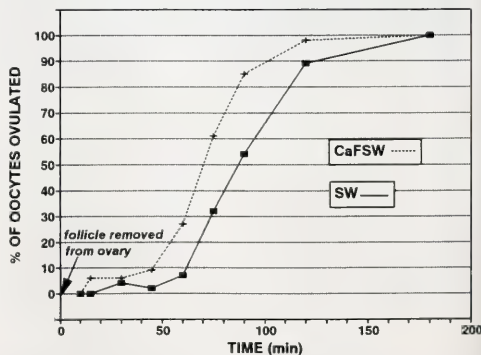


Figure 6. Typical kinetics of ovulation in oocytes obtained from the same ovarian lobes and subsequently incubated at 12° – 16°C in seawater versus calcium-free seawater. Note the accelerated rate of ovulation in CaFSW.

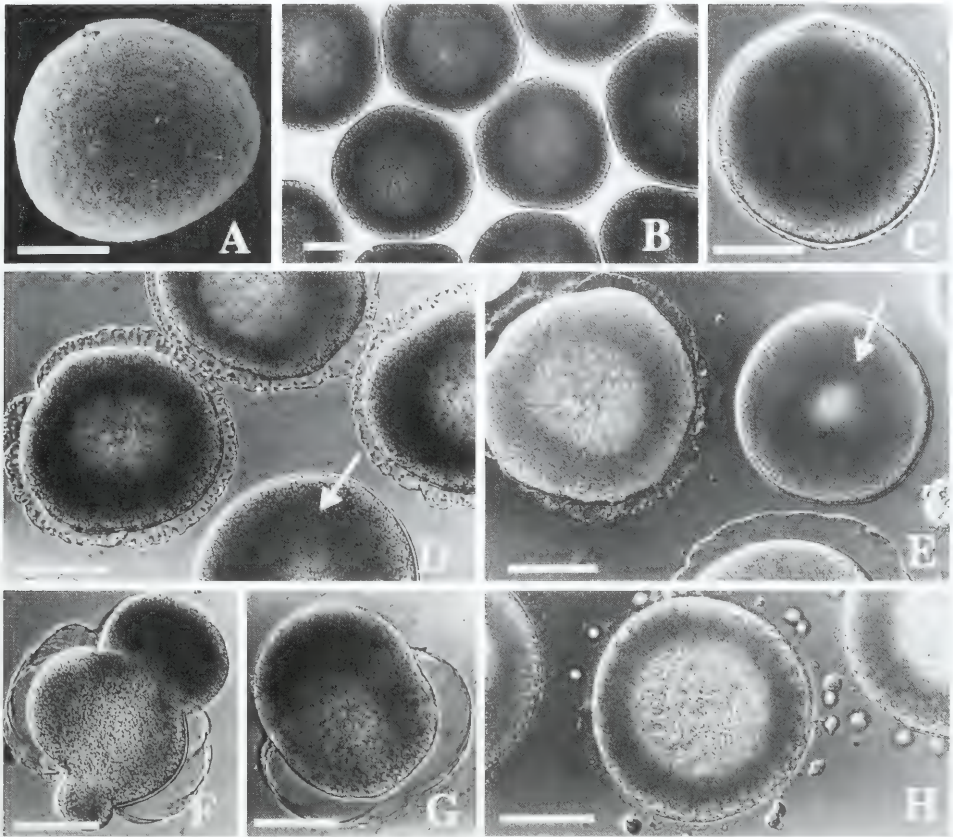


Figure 7. (A) SEM of an oocyte that had been mechanically stripped of its follicle cells about 15 min after removal of the follicle from the ovary and fixed at 20 min post-removal; scale bar = 50 μ m. (B) Photomicrograph of oocytes at 3 h after removal from the ovary. Such oocytes were mechanically stripped of their follicle cells 30 min after removal from the ovary and failed to undergo GVBD, as evidenced by the intact GV in each specimen; scale bar = 50 μ m. (C) Photomicrograph of an oocyte that was stripped of its follicle cells at 40 min after removal from the ovary and subsequently underwent normal GVBD; scale bar = 50 μ m. (D, E) Photomicrographs of specimens that were obtained from ovaries pre-incubated for 1–2 h in the gap-junction-uncoupling drugs heptanol (D) or α -glycyrrhetic acid (E) prior to ovarian maceration; most of the follicles obtained from such drug-treated ovaries and subsequently incubated in the drug failed to undergo GVBD by 3 h after removal of the follicles from the ovaries, although a few specimens (arrows) completed maturation; scale bars = 50 μ m. (F, G) Photomicrographs of drug-treated oocytes that had completed GVBD but that failed to ovulate normally after incubating for 3 h in the gap-junction-uncoupler heptanol (F) or α -glycyrrhetic acid (G); scale bar = 50 μ m. (H) Photomicrograph of cytochalasin-treated follicles that failed to undergo ovulation or GVBD by 3 h post-treatment, when ovaries were pre-incubated in 10 μ M CD for 1–2 h prior to the isolation of the follicles by ovarian maceration; compare with Fig. 4C, D where the ovaries were not pre-incubated in cytochalasin prior to maceration but the oocytes subsequently matured while in cytochalasin; scale bar = 50 μ m.

attached follicles. In *T. transversa*, however, seawater exposure by itself does not cause *in vitro* maturation, since oocytes stripped of their follicle cells less than

30 min after removal from the ovaries fail to undergo GVBD even when they are incubated overnight in seawater. Thus, the exposure to seawater, some other factor

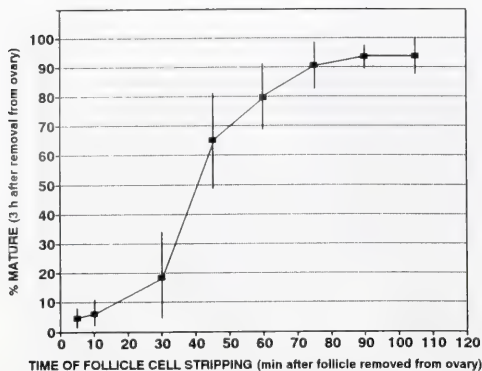


Figure 8. Mechanically removing follicle cells by means of a Nitex filter up to 30 min after obtaining the follicles from the ovaries yields low percentages of maturation by 3 h, whereas stripping follicle cells more than 40 min after obtaining the follicles from the ovaries does not significantly prevent maturation. These findings are consistent with the view that a maturation-inducing substance is transferred from the follicle cells to the oocyte within about 30–40 min after removing the follicle from the ovary. Vertical line = standard error of the mean; $n = 16$ for each time-point.

elicited by follicular detachment, or a combination of the two apparently triggers the production of an as-yet unidentified substance in the follicle cells, which in turn leads to oocyte maturation.

Although simple detachment of the follicle from the ovary seems capable of triggering oocyte maturation *in vitro*, a GSS may nevertheless function during normal spawnings in the field. Thus, in response to appropriate environmental cues such as daylength, temperature, or biomass load in the surrounding seawater, a GSS discharged by cells in the ovaries or adjacent tissues could trigger the detachment of follicles, perhaps by causing constrictions at sites where follicles connect to the germinal epithelium. Such a GSS-induced pathway would presumably take longer to detach follicles than does mechanical disruption, and could be fully replaced by the maceration procedure during *in vitro* trials. As indirect evidence for this view, overnight exposure of intact ovaries to an ovarian supernatant yields higher percentages of oocyte maturation than are observed in response to seawater alone, suggesting that some component of the supernatant helps to promote the detachment of the follicles in otherwise intact ovaries that are incubated for prolonged periods.

Ovulation-GVBD relationships and the roles of follicle cell-oocyte attachments during maturation

Following maceration of the ovaries, fully formed oocytes of *T. transversa* undergo ovulation and GVBD in

tandem. Thus, ovulated oocytes subsequently complete GVBD, and GVBD is not accomplished without prior shedding of the follicular sheath, suggesting that such processes may be causally linked. However, GVBD can be uncoupled from ovulation by experimental manipulations such as the application of cytochalasin D, which blocks follicle-cell capping but nevertheless allows GVBD to proceed. Conversely, if freshly collected specimens are mechanically stripped of their follicle cells within 30 min after removal from the ovary, GVBD typically fails to occur even though follicle cells are no longer present. Collectively, these findings suggest that although ovulation is normally followed by GVBD during *in vitro* maturation, successful removal of the follicular sheath is neither necessary nor sufficient to trigger GVBD in experimentally manipulated specimens.

The timing of ovulation can also be altered by CaFSW or trypsin treatments, which may simply accelerate follicle-cell capping by weakening follicle cell–oocyte attachments; incubating ovaries in seawater for more than 3 h prior to maceration may have the same effect. The converse of this mechanism could not, however, account for the more protracted ovulation obtained with oocytes from

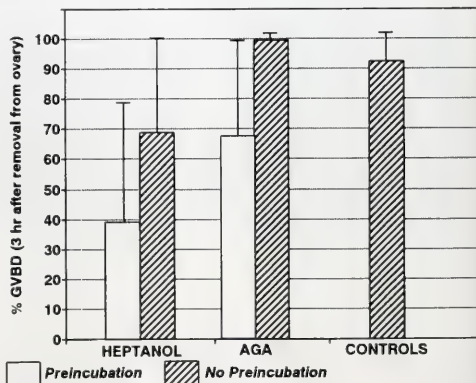


Figure 9. Pre-incubating ovaries in gap-junction-uncoupling drugs (1 mM heptanol or 25 μ M α -glycyrrhizic acid [= AGA]) for 1–2 h prior to ovarian maceration significantly reduces the percentages of maturation that are achieved after a subsequent 3-h treatment of the isolated follicles in the drug. Alternatively, follicles that are freshly obtained from macerated ovaries without a prior incubation in the drug before maceration do not show significantly lower percentages of maturation when subsequently incubated in the gap junction uncoupler for 3 h. Since the gap junction uncouplers require some time to disrupt intercellular communication, these findings are consistent with the view that functional follicle cell–oocyte attachments are required for about 30–40 min after follicular removal from the ovary in order for maturation to proceed. Controls are follicles isolated from the same ovaries as used for the drug treatments but not subjected to either of the gap-junction uncouplers. Vertical line = standard error of the mean; $n = 6$ for each treatment and control.

dorsal ovaries, unless oocytes of equal size in both types of ovaries somehow have more firmly attached follicle cells in the dorsal ovaries. Alternatively, separating the two shell valves removes the ventral ovaries from the lophophore and viscera, because these organs invariably remain attached to the dorsal valve. Oocytes obtained from separated ventral valves are thus no longer situated near the internal organs to receive the kinds of cues—either non-specific or specific—that apparently delay the ovulation of oocytes derived from isolated dorsal valves.

Evidence from experimental removal of follicular sheaths suggests that the follicle cells of *T. transversa* transport a maturation-inducing substance to the oocyte by about 30–40 min after the follicle is detached from the ovary. This timing also coincides with the kinetic data obtained from time-lapse video studies in that follicle cells are normally still attached to the oocyte 30–40 min after follicular removal from the ovary, and GVBD does not begin until about 70 min post-removal. Is the putative maturation-inducing substance actually transmitted from the follicle cells at around 30–40 min post-detachment of the follicle, or does an intact follicular sheath need to be continuously present for 30–40 min? These possibilities

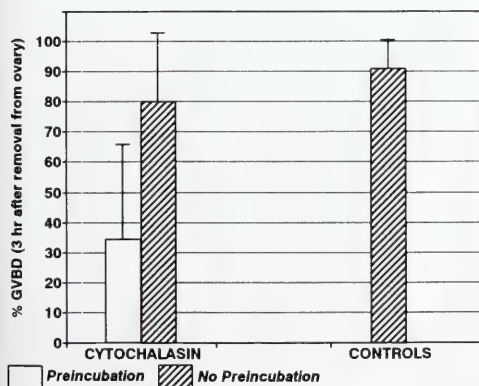


Figure 10. Preincubating ovaries in $10 \mu\text{M}$ cytochalasin for 1–2 h prior to ovarian maceration significantly reduces the percentages of maturation that are achieved after a subsequent 3-h treatment of the isolated follicles in the drug. Alternatively, follicles that are freshly obtained from macerated ovaries without a prior incubation in cytochalasin before maceration do not show significantly lower percentages of maturation when subsequently treated with cytochalasin for 3 h. Because cytochalasin treatments take about 1 h to alter follicle cell morphology, these findings are consistent with the view that functional follicle cell–oocyte attachments are required for 30–40 min after follicular removal from the ovary in order for maturation to proceed. Controls are follicles isolated from the same ovaries as used for the cytochalasin treatments but not subjected to cytochalasin. Bars = standard error of the mean; $n = 17$ for the “no pre-incubation” treatment, and $n = 7$ for the “pre-incubation treatment” and controls.

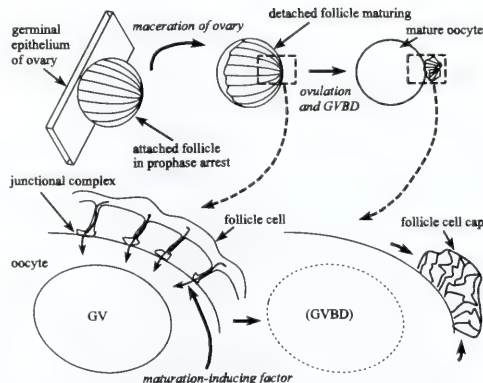


Figure 11. Diagram of the key role that follicle cell–oocyte attachments are believed to play during oocyte maturation, based on time-lapse video analyses and experimental manipulations.

cannot be distinguished on the basis of the experiments conducted in this investigation. However, unlike some other species in which a soluble maturation-inducing substance can be secreted externally to trigger maturation throughout the surface of the oocyte (Patino and Purkiss, 1993), in *T. transversa* the follicle cells apparently must be attached to the oolemma, judging from the fact that freshly collected oocytes that had been separated from their follicle cells did not undergo GVBD, even when kept in the fluids containing the stripped follicle cells. Similarly, gap junction uncouplers can at least partially prevent GVBD, even though follicle cells remain present near the non-maturing oocytes, following the apparent disruption of the follicle cell–oocyte junctions by the drugs.

The junctional complexes observed in *T. transversa* follicles have not been definitely identified as gap junctions, so the inhibitions obtained with the uncoupling drugs could be due to nonspecific effects rather than to actual disruptions of the junctions. In many cases, gap junctions can be distinguished by various ultrastructural characteristics, especially in the presence of lanthanum tracers (Larsen and Wert, 1989). Some invertebrate tissues, however, contain junctions that do not resemble conventional gap junctions but nevertheless allow small molecules to pass between neighboring cells (Germain and Anctil, 1996). Thus, unequivocal proof that the junctional complexes in *T. transversa* follicles possess the physiological properties of communicating junctions (as suggested by the drug tests) will necessitate functional analyses based on microelectrode investigations of ionic coupling (Gilula *et al.*, 1978) or dye-tracer studies using injected fluorescent probes (Browne *et al.*, 1979; Cerada *et al.*, 1993). Unfortunately, the vitelline envelope sur-

rounding *T. transversa* oocytes prevents easy access to the oolemma for electrophysiological recordings and hinders microinjection of fluorescent dyes by methods that have been successful with the oocytes of other marine invertebrates (Stricker *et al.*, 1992b, 1994b; Stricker, 1996).

In any case, the need for intact follicle cell–oocyte attachments is indirectly indicated by two other sets of results. First, gap-junction uncoupling drugs, which take about 30 min to block intercellular communications at 37°C (Downs, 1995), fail to block GVBD if *T. transversa* follicles are placed directly in the drug without preincubating the ovary prior to maceration. Maturation percentages are reduced, however, if the ovary is immersed in the drug for 1–2 h before maceration, particularly in the case of heptanol, which may use a more rapid mechanism of uncoupling than does AGA. Similarly, CD treatments typically fail to prevent GVBD when detached follicles are placed directly into the CD solution without an incubation in the drug before ovarian maceration. However, when ovaries are pre-incubated in CD, thereby allowing the drug more time to alter follicle-cell morphology before the ovary is macerated, CD significantly reduces GVBD levels. Thus, two very different kinds of drug treatments point toward the need for intact follicle cell–oocyte attachments and are consistent with the view that maturation can be inhibited if follicle cell–oocyte attachments are altered within 30–40 min after the follicle is detached from the macerated ovary.

Comparative biology of oocyte maturation in brachiopods

In the inarticulate brachiopod *Glottidia pyramidata*, oocyte maturation cannot be triggered by mechanical disruption of the ovaries, because such treatments tend to lyse oocytes (Freeman, 1994). Follicle-cell-free oocytes are obtained after repeatedly washing small pieces of *G. pyramidata* ovaries in CaFSW, but such oocytes fail to undergo GVBD (Freeman, 1994). It is not fully clear if this lack of maturation in the CaFSW-treated oocytes is simply due to a precocious removal of follicle cells prior to their delivery of a maturation-inducing signal, as has been observed in mechanically stripped follicles of *T. transversa*. However, following the CaFSW washes of *G. pyramidata* ovaries, “the follicular epithelium around the oocyte retracts” (Freeman, 1994, pg. 265), which implies that such CaFSW-treated specimens actively ovulate. Therefore, unlike the oocytes of *T. transversa*, which complete GVBD after ovulation, those of *G. pyramidata* are apparently capable of undergoing ovulation without concomitant GVBD.

Oocyte maturation can be elicited, however, if pieces of *G. pyramidata* ovaries are incubated with a soluble extract of the lophophore, although extracts from other tissues, including the gonads, fail to trigger GVBD (Free-

man, 1994). Similar incubations with membrane-permeant forms of cAMP also cause maturation, which suggest that the follicle cells of *G. pyramidata* possess a cAMP-based signaling pathway that is normally triggered by a lophophore-derived GSS to stimulate the secretion of a maturation-inducing substance (Freeman, 1994).

Whether *T. transversa* oocytes can be induced to mature in a similar fashion is difficult to determine because there is no reliable way of obtaining pieces of ripe ovaries without also triggering maturation through the mechanical detachment of the follicles. Hence, such studies would have to ensure that the experimental solutions gained access to the follicles within intact ovaries, and that maturation was not simply elicited by dislodging follicles from the ovaries. However, based on the fact that the simple maceration of *T. transversa* ovaries routinely triggers oocyte maturation, it follows that in this species (i) any GSS that might be produced by *T. transversa* is not restricted to the lophophore, and (ii) *in vitro* maturation does not require treatments with lophophore extracts or exogenously supplied cAMP, as has been described for *G. pyramidata*. The underlying causes for such apparent differences between the two species remain obscure.

Additional analyses of brachiopod ovulation and GVBD are essentially lacking, and the timing of these events relative to spawning and fertilization in the field are generally not known (Long and Stricker, 1991). The few observations that have been recorded indicate a range of possibilities. In the articulate brachiopod *Terebratulina retusa*, the follicle cells tend to be shed some time after spawning but apparently just prior to fertilization (James *et al.*, 1991a, b). In the inarticulate species *Crania anomala*, freshly spawned oocytes already lack follicle cells prior to fertilization (Nielsen, 1991). In contrast, spawned oocytes of the articulate brachiopod *Calloria inconspicua* retract their follicle cells after being fertilized (Chuang, 1996).

On the rare occasions when spawning has been observed in *T. transversa*, the oocytes contained an intact GV and a follicular sheath, but unlike what has been determined here for laboratory cultures, the exact maturation state of naturally spawned oocytes at the time of fertilization has not been ascertained (Long, 1964; Long and Stricker, 1991). Moreover, although long-term storage of adult *T. transversa* females in laboratory aquaria with running seawater often leads to an accumulation of fully grown, immature oocytes (Reed, 1987), the underlying mechanism for this apparent inhibition of spawning and maturation remains unknown. Thus, further comparisons between the laboratory microenvironment and the normal subtidal habitat may provide important clues about the natural stimuli for maturation (Rokop, 1977). Based on the *in vitro* analyses of *T. transversa* reported here, *in situ* investigations should consider the role that follicle

cells, and particularly their attachments to the oocyte, play in maturation and fertilization in the field.

Acknowledgments

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Egg-Mass Gel of *Melanochlamys diomedea* (Bergh) Protects Embryos From Low Salinity

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Abstract. Many opisthobranch gastropods embed their embryos in gelatinous egg masses; however, the functions of gel are not well known. We analyze the hypothesis that egg-mass gel protects embryos from salinity change. Using egg masses of *Melanochlamys diomedea*, we found that experimental removal of gel decreased the ability of embryos to survive osmotic stress. We evaluate several possible protective mechanisms by estimating osmotic influx of water into egg masses and by modeling salt efflux from an egg mass. On immersion in low-salinity water, egg masses lost roughly 23% of their mass, indicating that osmotic influx of water did not occur. Therefore, the principal route of salinity change within the egg mass is probably salt efflux. The model suggests that this efflux occurs quite slowly even when ambient salinity changes rapidly. Slow salinity change may be less stressful for embryos because the mechanisms that regulate cellular volume have more time to adjust. We show experimentally that slow salinity changes are less harmful to veligers than rapid ones by isolating capsules from egg-mass gel and exposing them to gradual or abrupt salinity change. The results support the hypothesis that rate of change of salinity is an important determinant of embryo survival and that egg-mass gel retards the rate of salinity change.

Introduction

Many marine invertebrates embed their embryos in gelatinous masses. Because gel eliminates convective transport within masses, it slows the exchange of material between embryos and seawater. Reduced exchange may adversely affect embryos. For example, embryos in the

centers of globose masses develop more slowly than those toward the edges, due to an inadequate supply of oxygen and the accumulation of waste (Booth, 1995; Chaffee and Strathmann, 1984; Strathmann and Strathmann, 1995). In other cases, however, reduced exchange may benefit embryos. For example, gel may retard the efflux of beneficial agents (salt water, heat) or the influx of damaging agents (fresh water, extreme heat, microorganisms). These kinds of benefits have been suggested, but little empirical work bears on them (Todd, 1981; reviewed by Pechenik, 1986). Gel may also protect embryos from predators and other environmental stresses, such as ultraviolet radiation, and may retain embryos in areas favorable for growth (Biermann *et al.*, 1992; Pechenik, 1986; Rumrill, 1990; Strathmann, 1985).

The egg masses of *Melanochlamys diomedea*, an opisthobranch gastropod, are particularly well suited to the study of the protective properties of gelatinous masses. Adult *M. diomedea* deposit masses in intertidal and shallow subtidal zones—on the bottoms of shallow, sandy lagoons and bays (Strathmann, 1987). These zones are exposed to a wide array of physical stresses. One common stress in the intertidal is low salinity, which may result from freshwater runoff (Drouin *et al.*, 1985; Sanders *et al.*, 1965) or rain (Gibbs, 1968; Tettelbach *et al.*, 1985). Low salinity can retard development or kill embryos, and even infrequent episodes of extremely low salinity may play an important role in the ecology and evolution of intertidal organisms.

This study is a four-part experimental and theoretical analysis of the hypothesis that egg-mass gel protects embryos from salinity change (Pechenik, 1982, 1983; Skoog, 1973; Thorson, 1946; Todd, 1981). (1) We tested the protective ability of the gel by taking advantage of egg-mass structure. Embryos are contained in capsules that are distributed throughout a gelatinous matrix; the cap-

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sules are easily removed from the gel by agitation. We exposed both embryos in gel and embryos removed from gel to a range of salinities and measured their survival. (2) We estimated the magnitude of osmotic influx of water into egg masses exposed to low-salinity water. (3) We evaluated several potential mechanisms of protection (Pechenik, 1983) by adapting a model of diffusion in a sphere (Crank, 1956) to salt efflux within and from egg masses. The model suggested several ways that gel may protect embryos from osmotic stress. One model prediction was that gel slows the rate of salinity change. (4) Using embryos isolated from gel, we experimentally tested the hypothesis that slow salinity change is less harmful than abrupt salinity change (Milne, 1938; Pechenik, 1982, 1983; Sanders *et al.*, 1965; Stickle and Ahokas, 1974). We discuss how our findings about salinity may apply to other substances that move into and out of egg masses by diffusion, and how transport considerations may bear on egg-mass design.

Materials and Methods

Egg masses

Opisthobranch egg masses consist of many clear capsules embedded within a gelatinous material, each capsule containing one to several embryos (Hurst, 1967). A typical *M. diomedea* egg mass is globose (Hurst, 1967), contains about 25,000 to 50,000 embryos (Lee and Strathmann, in press), and is tethered to the sediment by a strand of gel.

Egg masses were collected from False Bay, San Juan Island, Washington. The collection site is fed by a freshwater creek that may dramatically lower the salinity within the bay. During a low tide in April 1997 (after a period of rain), we measured a salinity of 0‰ near the middle of the bay. In the same area, we found four egg masses in which all embryos had died. Later in the *M. diomedea* breeding season (summer, fall), the creek flow rate is generally lower but may increase after rains.

Egg masses for experiments were collected during low tides in June 1995 and April and July 1997. We picked masses that had been laid recently, avoiding masses that were ragged, covered with diatoms, or in the late stages of hatching. Within 2 h of collection, the masses were placed in flow-through seawater tables (average temperature 11°C) at Friday Harbor Laboratories, San Juan Island. Several airstones were placed in the water near the masses to ensure adequate aeration. During experiments, all manipulated and unmanipulated egg masses were kept in small Pyrex or plastic bowls submerged in seawater in the tables.

Comparison of gelled and degelled egg capsules

All seawater used in the experiments was bag-filtered (10- μ m pore size), and test salinities were diluted with

fresh water purified by reverse osmosis. Salinities were measured with a hand-held refractometer ($\pm 1‰$). Undiluted seawater (30‰) served as the control. The survival of embryos in unmanipulated egg masses and of embryos that had been removed from the gel was determined at five test salinities (0, 5, 10, 15, 30‰). We tested 18 egg masses, 9 degelled and 9 intact, at each of the five salinities, for a total of 90 masses.

To minimize age effects, egg masses for the experiment were limited to those containing relatively young embryos (pre-veligers without red "kidney spot," velum, or shell). To remove embryos from an egg mass (the "degelled" treatment), we cut the mass into small pieces, placed the pieces in a small glass vial containing 3 ml of seawater, shook the vial vigorously for 30 s, and filtered its contents through a 200- μ m mesh (Nitex). The mesh retained large pieces of gel but allowed dislodged egg capsules to pass. For a subset of the manipulated egg masses, we checked egg capsules under a dissecting microscope (20 $\times$) to ensure that they were completely disaggregated and free of gel.

Immersion into the test salinity was abrupt. Capsules were poured onto a 64- μ m mesh (Nitex), briefly rinsed with water at the test salinity, and washed into 50 ml of test water in a small Pyrex or plastic bowl. We patted dry the unmanipulated egg masses and placed them into 50 ml of the test water. Each bowl was agitated several times during the experiment to reduce boundary layers. After 3 h we transferred capsules and masses back to full-strength seawater by similar processes. Each egg mass, or the capsules from an egg mass, was counted as a replicate.

Two additional controls were necessary. First, the pH of fresh water (6.9; measured with a microelectrode) was lower than that of seawater (7.9). The seawater-dilution and freshwater treatments (15, 10, 5, 0‰) therefore experienced lower pH (7.9, 7.8, 7.6, 6.9, respectively). Low pH may adversely affect the development of some marine invertebrate larvae (Calabrese and Davis, 1966; Strathmann and Strathmann, 1995). To test the effects of pH, we brought some seawater to pH 6.9 with concentrated HCl. We degelled capsules of four additional masses, placed them in the acidic seawater for 3 h, then returned them to normal seawater. Second, the method used for separating egg capsules from the egg mass involves vigorous agitation, which may damage embryos so that they are more susceptible to osmotic stress. To test the effects of agitation, we degelled 15 masses by the method described above and 15 masses by a gentler method in which each mass was cut into small pieces and washed with a small amount of seawater. Dislodged capsules were picked out with a pipette. Capsules from each separation treatment were divided into three groups (capsules from five masses in each), and groups were subjected to 5, 10, or 30‰ by the method described above. After 3 h in the test salinity, capsules were transferred back to seawater.

Three days later we determined the survival rate for each egg mass. To standardize the scoring of manipulated and unmanipulated masses, we removed the egg capsules from the gel in the unmanipulated groups by the process described above. To score embryos as alive or dead, we examined 50–100 capsules from each mass under a compound microscope (100 $\times$). At this time almost all embryos in the control groups had become veligers (had developed red kidney spots, shells, velar lobes). We scored as alive those veligers having a red kidney spot and shell, showing no major deformities, and actively moving within the egg capsule. Dead individuals lacked several of the above characteristics and showed substantial tissue damage or loose debris in the egg capsule.

Movement of water between egg mass and environment

Fresh water may affect egg-mass size and shape by changing gel structure or by moving osmotically into the egg mass. We tested these possibilities by immersing 12 egg masses (collected in April 1997) in low-salinity water (5‰) for 3 h, and weighing them at 0, 10, 20, 30, 60, 90, 120, and 180 min. At each weighing, masses were blotted briefly and weighed to the nearest milligram. After 180 min, masses were returned to seawater and were weighed once more about 10 h later. Control masses were weighed on the same schedule but were kept in seawater for the duration of the experiment.

To examine more closely the mechanisms of weight change in egg masses, we performed a separate experiment to measure both fresh and dry weights of masses exposed to a variety of salinity conditions. Forty egg masses (collected in July 1997) were assigned haphazardly to four groups of ten. One group was exposed to low-salinity water (5‰) for 3 h, another to seawater for 3 h, another to low-salinity water for 3 h followed by seawater for 10 h, and another to seawater for 3 h followed by seawater again for 10 h. Masses were weighed (as described above) before and after the treatments, dried for 3 days at 60°C, and reweighed.

A model of salinity change at different locations in an egg mass

The non-steady-state diffusion of a substance from a sphere is described by the equation (Crank, 1956, p. 86)

$c(r, t)$

$$= c_i + (c_0 - c_i) \left(1 + \frac{2a}{\pi} \sum_{n=1}^{\infty} \frac{(-1)^n}{n} \sin \frac{n\pi r}{a} e^{-Dn^2\pi^2/a^2} \right)$$

where $c(r, t)$ is the concentration of diffusing substance at a distance r from the center of the egg mass at time t , c_i is the initial uniform concentration of substance, c_0 is the

surface concentration, a is the radius of the sphere, and D is the diffusion constant of the substance. We adapted this equation by making several assumptions: (1) Egg masses are spherical with $a = 0.566$ cm (see Results). This is not entirely accurate. Masses are on average 2.4 times longer than wide, which will result in model predictions that slightly underestimate the true rate of salinity change. (2) Seawater consists solely of sodium chloride and water. (3) The diffusion constant of sodium chloride, D , is the same in the gelatinous matrix as in water. We estimated D of sodium chloride in water (which diffuses as Na^+ and Cl^-) at 12°C as $1.043 \times 10^{-5} \text{ cm}^2/\text{s}$ (calculated from data in Lobo and Quaresma, 1989). The actual value of D may be lower, especially if the gelatinous matrix hinders movement of ions (or if ions adsorb onto the gel material). However, measured values of the diffusion constant of oxygen in *M. diomedea* egg masses are close to those of oxygen in water (Cohen and Strathmann, 1996). If D for sodium chloride is lower in the gel, the model will overestimate the rate of change of salinity. (4) Embryos and capsules do not affect diffusion through the mass. Gelatinous matrix makes up the largest fraction of the mass, approximately 93% by volume for freshly laid masses (calculated from unpublished data of C. E. Lee, University of Washington). This fraction probably changes somewhat as embryos develop, because both capsules and egg masses swell (Kress, 1971; H. A. Woods, pers. obs.). Even in late-stage egg masses, however, capsules are well-spaced (H. A. Woods, pers. obs.). If embryos did affect the diffusion constant, they would increase it (Hunter and Vogel, 1986)—leading us to underestimate the true rate of salinity change. (5) Masses experience no shape change or osmotic influx of water, only efflux of salt. If fresh water flows osmotically into masses (see Discussion), rates of salinity change will be underestimated. (6) Masses are in a well-stirred environment that reduces boundary layers to insignificance.

We wrote a computer program to calculate $c(r, t)$. The solution to $c(r, t)$ consists of an infinite series of terms, indexed by n . We approximated the solution by iterating each determination to $n = 1000$, which was sufficient for the calculation to converge to within a small fraction of a percent of the actual solution (checked in a subset of cases by iterating to $n = 10,000$). The profile of salinity change was calculated for a mass initially at 30‰ subjected abruptly to 5‰ ($c_0 = 5$). The effects of egg mass size (a) and distance from the center of the mass (r) were explored.

To determine the value of a (egg-mass radius), we measured 31 egg masses. Mass tethers were removed and the masses were laid on a petri dish. We measured lengths with a ruler (± 1 mm) and circumferences at the widest point with a piece of string which we then measured with a ruler (Booth, 1995). After blotting each mass dry,

we weighed it (± 0.01 g) and determined its volume (± 0.05 ml) by the displacement of 5 ml of seawater in a 10-ml graduated cylinder (Booth, 1995).

Effects of gradual versus abrupt salinity change

We compared the survival of degelled embryos exposed to abrupt and gradual salinity changes. Ten egg masses were degelled, five with pre-kidney-spot embryos and five with kidney-spot veligers. The capsules from each of these masses were split into three groups: one group maintained in full-strength seawater for the duration of the experiment; a second group exposed abruptly to 5‰ for 1 h by the method described above; and a third group exposed to a gradual decline in salinity before exposure to 5‰ for 1 h. Egg capsules of the third group were initially in 5 ml of water in a 25-ml glass vial. Four times at intervals of 20 min we added 5 ml of fresh water to the vials. After each step, the measured salinity in the vial deviated from the calculated salinity by less than 1‰. Capsules were left at 5‰ for 1 h. During this time, after the capsules had settled to the bottom of the vial, we removed all except 5 ml of water. We increased the salinity to full strength (30‰) over 20 min in three steps by adding 1.65 ml and then 11.65 ml of full-strength seawater, then finally filtering the capsules into seawater. We scored individuals 6 days later, by which time all embryos had become veligers or died.

Results

Gelled versus degelled capsules

The effect of different salinities on gelled and degelled capsules is shown in Figure 1. The fraction of embryos

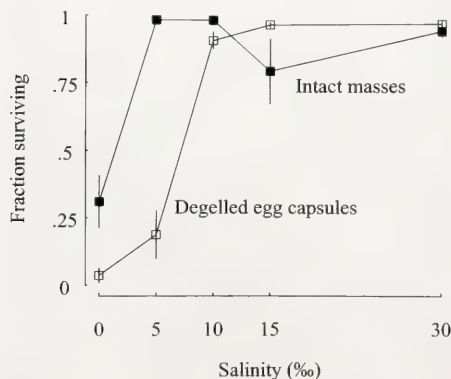


Figure 1. Survival of *Melanochlamys diomedea* embryos after immersion in various salinities for 3 h. Survival was scored 3 days after exposure to the test salinity. Filled squares (■) indicate embryos in egg masses, open squares (□) degelled embryos.

Table 1

*Analysis of variance summary of the data, presented in Figure 1, for survival of *Melanochlamys diomedea* embryos at various salinities*

Source	SS	df	MS	F	P
Salinity	15.99	4	4.00	57.3	<0.001
Egg-mass treatment (intact or degelled)	1.89	1	1.89	27.4	<0.001
Salinity * Mass trt.	4.87	4	1.22	17.64	<0.001
Residual	5.52	80	0.07		

surviving in all groups was uniformly high at 10‰ or greater. In the intact groups, the fraction surviving was also high at 5‰ (0.98) and only dropped off at 0‰ (0.31). In the degelled groups, survival was lower at 5‰ (0.19) and very low at 0‰ (0.04). At 5 or 10‰, cellular debris and internal parts were visible in the intracapsular fluid. In fresh water, the majority of tissues were severely degraded.

These results indicate that gel is not necessary for protection at salinities above 10‰, assuming a 3-h exposure. The gel improved survival only when the salinity dropped below 10‰, but in this range the improvement was marked. The exceptionally large error bars on the gelled group at 15‰ were due to two probable outliers (survival of 0.24 and 0.06). It is possible that the embryos in these masses had died before they were collected. The data were transformed (angular) and differences were tested with a factorial analysis of variance. Both of the main terms (salinity and egg-mass treatment) were highly significant (Table 1). In addition, the interaction term was significant, indicating that low salinity killed the degelled group disproportionately (Fig. 1). The salinity that killed 50% of the embryos in each group—the LD50 ($\pm 95\%$ CI)—was calculated with logistic regression (Venables and Ripley, 1994) after removal of the two outliers and the 30‰ groups. The LD50 of the degelled group ($6.95 \pm 0.20\%$) was higher than that of the embryos in intact masses ($1.17 \pm 0.05\%$).

Embryos exposed to low pH survived well (approx. 0.98), indicating that the lower pH of the more dilute groups in the main experiment did not confound the results. In addition, vigorous agitation did not make embryos more susceptible to osmotic stress. At the three test salinities (5, 10, and 30‰) of the control experiment, embryos in the agitated group survived at essentially the same rate (mean 0.04, 0.99, 1.0, respectively) as embryos in the "gentle" group (mean 0.02, 0.99, 0.99, respectively).

Water movement

Egg-mass weight declined rapidly during the first half hour of exposure to low-salinity water (5‰), then less

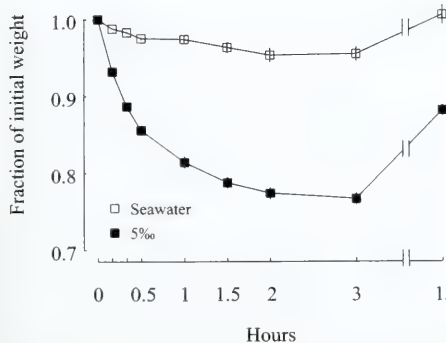


Figure 2. Fraction of *Melanochlamys diomedea* egg-mass weight remaining after various exposures to low-salinity water (5‰, ■) or seawater (□). After 3 h, the low-salinity masses were returned to seawater.

slowly before reaching a more-or-less constant level of about 77% of the initial weight (Fig. 2). During the subsequent 10 h in seawater, the masses regained much of their weight (88% initial weight). The control masses, in seawater for the duration of the experiment, also lost some weight (<5%) over 3 h, most likely by losing small bits of gel during blotting. The weights of the control egg masses also increased after 10 h, probably reflecting a gradual swelling during development (Kress, 1971; H. A. Woods, pers. obs.). In no case did an egg mass undergo any readily apparent change in shape.

A second experiment confirmed that egg masses exposed to low salinity lost wet weight, and that the weight was regained during subsequent exposure to seawater (Table II). In addition, low salinity affected dry weight. Egg masses exposed to low salinity for 3 h had a mean dry weight (0.005 ± 0.001 g) roughly a third of the mean dry weight of masses exposed to seawater for 3 h (0.015 ± 0.001 g). Masses exposed to low-salinity water for 3 h followed by seawater for 10 h, however, had a mean dry weight (0.017 ± 0.002 g), similar to the two other groups

of masses exposed only to seawater (0.015 ± 0.001 and 0.019 ± 0.003 g).

Model and morphology

Table III shows the results of the morphological measurements of 31 egg masses. Together, average weight (0.75 g) and average volume (0.76 ml) indicate that the egg masses were nearly the same density as water. Although not spherical, the masses were only 2.4 times longer than wide. For the model, we estimated the radius, $a = 0.566$ cm, as the radius of a sphere having the same volume as the average measured volume (0.76 cm^3). We note that the masses (collected in early June) used for these measurements (Table III) were substantially larger than the masses collected (in late July) for the second water-movement experiment (Table II).

Figures 3 and 4 illustrate some model predictions. The salinities at three positions in an egg mass (near the middle, $r = 0.1$ cm; about halfway between center and edge, $r = 0.3$ cm; and near the edge, $r = 0.5$ cm) after 3 h of immersion in low salinity (5‰) were all predicted to be between 5 and 10‰ (Fig. 3). This range is one of high sensitivity for the embryos (Fig. 1); the model suggests that embryos near the middle ($r = 0.1$ cm) may have experienced slightly more tolerable salinities than embryos near the edge ($r = 0.5$ cm). The rate of change of salinity also depended on the position within the egg mass, with embryos near the edge experiencing higher rates. However, at all positions, the rate of change below 10‰ was quite slow.

The model also predicted that egg-mass size affects the rate of change of salinity (Fig. 4). Not surprisingly, the salinity in an egg mass 25% larger (by volume; $a = 0.609$ cm) changed more slowly than in one 25% smaller ($a = 0.514$ cm; salinity was modeled at $r = 0.3$ cm in both). However, the effect of a 25% increase or decrease in egg-mass volume was not as dramatic as the effect of position within the egg mass (Fig. 3).

Gradual versus abrupt salinity change

Both salinity treatment and embryo age significantly affected the fraction of embryos surviving (Fig. 5; 2-

Table II

Summary of egg-mass weights before and after exposure to low-salinity water (5‰), seawater, or both

Treatment	Initial fresh weight	Fresh weight after treatment	Dry weight after treatment	Dry weight as % of initial weight
3 h in seawater	0.37 ± 0.05	0.38 ± 0.05	0.015 ± 0.002	4.1 ± 0.1
3 h in 5‰	0.33 ± 0.05	0.28 ± 0.04	0.005 ± 0.001	1.4 ± 0.1
3 h in seawater + 10 h in seawater	0.36 ± 0.04	0.41 ± 0.05	0.017 ± 0.002	4.8 ± 0.1
3 h in 5‰ + 10 h in seawater	0.42 ± 0.07	0.45 ± 0.07	0.019 ± 0.003	4.7 ± 0.1

All weight are given in g $\pm$ SEM except last column (%). For each treatment, $n = 10$.

Table III

Morphology of Melanochlamys diomedea egg masses (n = 31)

	Mean	SEM	Range
Length (cm)	2.25	0.32	1.5–3.8
Circumference (cm)	2.96	0.18	2.3–3.6
Volume (ml)	0.76	0.28	0.35–1.30
Mass (g)	0.75	0.29	0.34–1.32

factor ANOVA on angularly transformed data: $F = 37.9$, $P < 0.0001$ and $F = 40.6$, $P < 0.0001$, respectively). All embryos survived the control treatment well. Age affected the response to gradual and abrupt treatments. Veligers (dashed lines) survived better overall and also survived the gradual change (mean = 0.80) better than the abrupt change (0.30). The younger, pre-kidney-spot, embryos did not survive well under either the gradual (0.07) or abrupt (0.01) conditions of change. We note that, because individuals in different treatments were not completely independent (capsules within a mass were split into groups), ANOVA is not an entirely appropriate statistical test; however, no better test is available.

Discussion

Our observations demonstrate that gelatinous egg masses can protect embryos from salinity changes (Fig. 1). Although isolated embryos survived 3-h exposures to a wide range of salinities (10–30‰), gel markedly improved survival at the lowest salinities (0, 5‰). Gelatinous masses

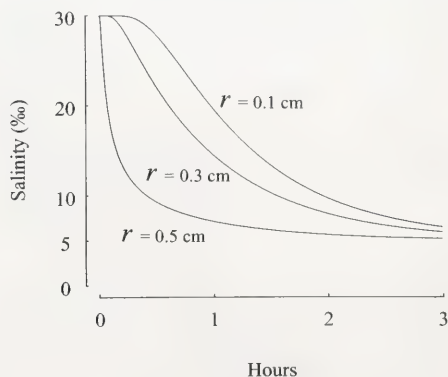


Figure 3. Model predictions of *Melanochlamys diomedea* egg-mass salinity as a function of time at three internal positions. The egg mass was assumed to be spherical with radius $a = 0.566$ cm. At the beginning of the simulation the salinity was 30‰ throughout the egg mass. The external salinity was held constant at 5‰.

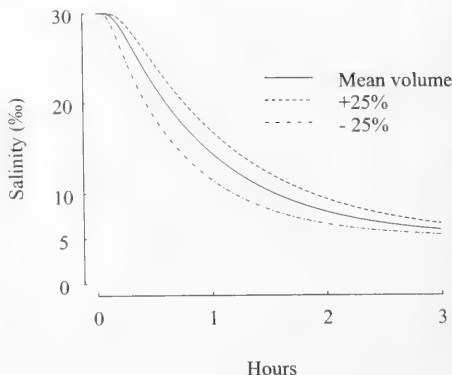


Figure 4. Model predictions of salinity change in egg masses of *Melanochlamys diomedea* as a function of time at a position about halfway between the center and the edge ($r = 0.3$ cm) of a spherical egg mass ($a = 0.566$ cm). The dashed lines indicate the trajectories at this same position ($r = 0.3$ cm) in a mass 25% larger ($a = 0.609$ cm) or smaller ($a = 0.514$ cm).

have not been previously examined for this protective ability, with the exception of the egg masses of a polychaete, *Scoloplos armiger* (Gibbs, 1968). The results parallel those presented here. Polychaete larvae within intact masses and those removed from egg masses survived sudden immersion in salinities as low as 14.4‰ (Gibbs, 1968). However, at an immersion salinity of 7.7‰, only polychaete larvae

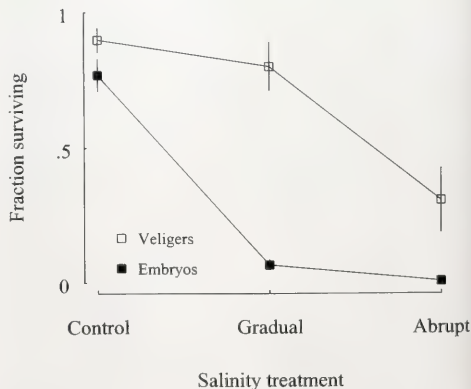


Figure 5. Survival of degelled embryos (■) and veligers (□) of *Melanochlamys diomedea* subjected to gradual or abrupt salinity change. Control refers to groups maintained in seawater for the duration of the experiment; gradual to embryos ramped to low salinity (5‰); and abrupt to embryos immersed immediately into low salinity (5‰).

inside the egg masses survived (0.67). No larvae survived immersion in fresh water.

Pechenik (1983) has suggested four possible mechanisms by which egg masses might protect embryos from salinity changes. Egg masses might (1) prevent the internal salinity from declining; (2) maintain an equilibrium concentration higher than ambient; (3) reduce the time spent at low salinity; (4) reduce the rate of change of salinity. The first and second hypotheses are unlikely in view of the mass structure. Gel consists predominantly of water in a matrix of mucopolysaccharides (Ghiselin, 1965; Todd, 1981), which would be unlikely to retain small solutes indefinitely. It is possible, however, that the matrix retains large, osmotically active molecules (Pechenik, 1983), or that the mucopolysaccharides themselves are osmotically active.

The third and fourth hypotheses may apply to *Melanochlamys diomedea*, but only if salinity within egg masses changes slowly. Our observations strongly suggest that this is the case. Egg masses do not experience a rapid osmotic influx of water. A rapid influx would cause egg masses to swell and gain weight; in contrast, we observed that the egg masses lost weight during the 3-h exposure to low salinity (Fig. 2). Replacement of high-density seawater by low-density, low-salinity water cannot explain these results, as the densities of seawater and low-salinity water differ by only about 2% (Horne, 1969). The weight loss suggests instead that, on contact with low-salinity water, the gel contracted and expelled some internal water—perhaps because the change in water activity altered the conformation of the gelatinous matrix (Parsegian *et al.*, 1995). An alternative explanation, breakdown and loss of the gel itself, is unlikely for several reasons. First, the difference in dry weights between egg masses exposed to seawater or low-salinity water (5‰) (Table II) can be accounted for largely by the difference in the salt content of seawater and low-salinity water. An egg mass weighing 0.38 g (fresh) would contain about 8 mg of salt if the internal water were seawater; a similar mass in low-salinity water (5‰)—assuming that it had shrunk to 77% its initial volume (Fig. 2)—would contain about 1 mg of salt. The difference between these values, 7 mg, is the larger part of the difference between the dry weights of masses exposed to seawater and low-salinity water for 3 h (10 mg, Table II). Second, comparison of the second and fourth treatments (Table II) shows that virtually all of the dry mass lost after a 3-h exposure to low salinity was regained during a subsequent exposure to seawater, an observation interpreted most simply as the influx of salt into an otherwise intact gelatinous matrix.

Because osmotic influx of water is not important, the principal mechanism of salinity change within the egg mass must be the efflux of salt. The model indicates that this efflux occurs quite slowly, especially as the concen-

tration gradient becomes small (as the internal salinity falls below 10‰). The loss of salt will be accelerated somewhat by the decrease in the size of the egg mass (Fig. 2). In addition, smaller egg masses will experience higher rates of internal salinity change. However, as shown in Figure 4, even a 25% decrease in mass volume does not substantially change the trajectory of salinity change. Furthermore, we assumed that salt diffuses in gel as rapidly as it does in water. If salt diffuses more slowly in gel—a distinct possibility (see model assumptions above)—then the salinity would change even less rapidly than the model suggests.

Therefore, Pechenik's third hypothesis (above) may apply to *M. diomedea*. The maximum exposure to low salinity is set by the duration of the tidal cycle, about 6 h (exposure will be considerably shorter than this at sites close to the low tide mark). The model (Eq. 1, Figs. 3, 4) suggests that an egg mass requires more than 3 h to equilibrate with ambient salinities. In nature, therefore, the internal salinities of gel masses may decrease slowly enough that incoming tides rescue them before they equilibrate with low ambient salinity.

The fourth hypothesis (above) gains additional support from our experiments on veliger survival. We showed that veligers within egg masses survived a slow rate of salinity change better than an abrupt change (Fig. 5). We note that only veligers benefited from the gradual change (Fig. 5); embryos fared equally poorly in both gradual and abrupt changes. This may be due to the veligers' well-developed shells and opercular membranes (Thorson, 1946), which when closed, would retard the movement of solutes and water. Embryo survival may be improved by even slower salinity changes, especially between 10 and 5‰.

Why is the rate of change of salinity important to embryos? As salt leaves the egg mass, and the interior salinity falls, embryos will inhabit an increasingly hypoosmotic environment. Consequently, water will flow osmotically from the gelatinous matrix into the embryonic cells. The principal danger from this influx of water is cell swelling, which may inhibit cell function or, in extreme cases, damage or burst cells. Although intracellular solutes were not studied in *M. diomedea*, many marine invertebrates regulate the levels of these substances, especially free amino acids and some inorganic ions, in order to minimize osmotic concentration differences between cellular and external spaces (Amende and Pierce, 1980; Bedford, 1971; Chamberlin and Strange, 1989; Clark, 1985; Florkin and Schoffeniels, 1969; Greenwalt and Bishop, 1980; Kasschau, 1975; Pierce and Amende, 1981; Silva and Wright, 1994). But regulation of intracellular solutes is not instantaneous (Amende and Pierce, 1980; Burton, 1991; Neufeld and Wright, 1996; Silva and Wright, 1994). We speculate that regulation of cell volume operates over

the scale of minutes to hours in *M. diomedea*, and that egg mass gel protects by slowing the rate of salinity change so that cellular mechanisms of volume regulation are not overwhelmed.

Pechenik (1982, 1983) has examined another encapsulating structure, the egg capsule of prosobranch gastropods, for its ability to protect embryos from salinity change. Here the term "egg capsule" refers not to a structure similar to *M. diomedea* egg capsules (thin, permeable capsules embedded within the gel), but to a tough, leathery, semipermeable capsule that encloses both intracapsular fluid and embryos. Although capsule walls are thin (56 and 109 μm for two species of *Nucella*), they effectively protected embryos from osmotic changes comparable to those in this study (Pechenik, 1982). Considering the mechanisms listed above, Pechenik (1983) concluded that the second and fourth were likely to be important. The intracapsular osmotic concentration equilibrated to 25–50 mOsm/l above ambient osmotic concentration, and a gradual osmotic change improved the survival of embryos that had been removed from their capsules. Thus, while prosobranch capsules and opisthobranch masses both protect embryos from osmotic change, the mechanisms may be different: prosobranch capsules resist salinity change with semipermeable membranes, whereas opisthobranch masses resist by retaining relatively large volumes of water and exploiting the slowness of diffusion.

A challenging problem in the study of taxa that lay gelatinous egg masses is to explain the interspecific diversity of egg-mass sizes and shapes, which range from thin sheets or coils to small, tethered spheroids to large, sausage-shaped masses (Booth, 1995; Hurst, 1967; Strathmann, 1987). Our findings, and those of others, suggest that gel has a profound effect on exchange processes between the interior of the egg mass and the environment. For example, embryonic oxygen requirements may impose an upper limit on the size of egg masses (Strathmann and Strathmann, 1995; but see Booth, 1995). An egg mass optimized for oxygen delivery should therefore be relatively small. On the other hand, the present study suggests that smaller masses are less able to protect from salinity change. The size and shape of egg masses may be determined in part by compromises between maximizing rate of oxygen delivery and maximizing protection from salinity change. The importance of various exchange processes will depend on the environment (for example, subtidal species may not be affected by salinity changes). Of course, other gel functions not related to exchange, such as protection from predators, retention of embryos in favorable sites, and protection from ultraviolet radiation, may also be important (Biermann *et al.*, 1992; Pechenik, 1986; Rumrill, 1990; Strathmann, 1985). Further physiological, ecological, and comparative studies will be

necessary to determine the actual importance of various gel functions.

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Myogenic Heartbeat in the Primitive Crustacean *Triops longicaudatus*

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Abstract. Pacemaker mechanisms in the heart of the primitive crustacean *Triops longicaudatus* were examined electrophysiologically. The heart is tubular and the heart wall consists of a single layer of myocardial cells. No nerve cells were found in the heart, either with methylene blue vital staining or by light microscopy of serial sections. The heart beats rhythmically at a frequency of 120 to 240 beats/min, and each beat is associated with a slow membrane potential change in the heart muscle. The amplitude of the slow potential varies widely and no spikes appear on it. The heart muscle cells are electrically coupled with each other and generate synchronous slow potentials. No localized portion of the heart exhibited a frequency that always preceded the others. The muscle activity could be phase-shifted by injection of a single brief current pulse and could be entrained to a lower or higher frequency by repeated brief current pulses injected into the muscle cell. The frequency of muscle activity could be changed by the injection of DC current into the muscle cell, and the change in frequency was linearly related to the intensity of the current. When the intensity of hyperpolarizing DC current exceeded a certain value, the muscle activity disappeared abruptly, and the heart stopped beating completely. These results show clearly that the heartbeat of *Triops* is myogenic. The heart is diffusely myogenic and should be regarded as a single muscle oscillator.

Introduction

The heartbeat of many crustaceans is neurogenic (reviewed by Krijgsman, 1952; Maynard, 1960; Prosser,

1973). The cardiac ganglion situated in the heart acts as a pacemaker. Neurogenic heart muscle has no endogenous rhythmical properties and is driven by periodic bursting activity of the cardiac ganglion via excitatory neuromuscular junctions. However, some diversity in the pacemaker mechanism of crustacean hearts has been reported recently. Yamagishi (1996) and Yamagishi and Hirose (1997) have shown, in the isopod *Ligia exotica*, that the heart of embryos and early juveniles exhibits myogenic heartbeats; i.e., the activity in the myocardium arises endogenously. Later, as the juveniles develop, the cardiac ganglion initiates its spontaneous bursting activity and begins to act as a primary pacemaker, entraining the muscle activity via neuromuscular excitatory junctional potentials. This type of late-developing neurogenic heartbeat is different from that in any other known crustacean.

The heartbeat of branchiopods, one of the lower orders within the Crustacea, is said to be myogenic (reviewed by Krijgsman, 1952; Maynard, 1960). This idea was proposed on the basis of the following two observations. First, no nerve cells were found in the heart of *Daphnia* (Ingle, cited by Prosser, 1942). Second, the heartbeat of *Daphnia* was suppressed by external application of acetylcholine to the body, which was thought to suppress myogenic heartbeats (Baylor, 1942; Bekker and Krijgsman, 1951). However, the second observation is not now regarded as evidence of a myogenic heartbeat, because acceleration of the myogenic heartbeat by acetylcholine has been reported (e.g., Greenberg, 1965). Thus, the cardiac pacemaker mechanism of branchiopods is still uncertain.

To explore the diversity in the heartbeat pacemaker mechanisms of crustaceans, we examined the electrophysiology of the heart of the primitive crustacean *Triops longicaudatus* (Notostraca, Branchiopoda). The aim was to determine whether the heartbeat is myogenic. The results show clearly that the heartbeat of *Triops* is myogenic, and that the heart should be regarded as a single

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muscle oscillator. Some of these observations have appeared in abstract form (Yamagishi *et al.*, 1993).

Materials and Methods

Animals

Adult specimens of the freshwater tadpole shrimp *Triops longicaudatus* have been collected routinely in June from rice fields in Saitama, Japan. They were kept in a freshwater aquarium in the laboratory, at room temperature, for about 3 weeks. Over a hundred specimens, 15 to 26 mm in body length, were used for the experiments.

Morphology

Morphological observations of the heart were made in dissected animals by vital staining with methylene blue, or by light microscopy in serial sections of the heart. For sections, hearts that had been isolated together with the dorsal body wall were fixed in aqueous Bouin's solution. Then, the preparations were dehydrated in a graded ethanol-*n*-butanol series, embedded in paraffin, and cut transversely into serial sections (5 to 7 μ m). The sections were stained for light microscopy with Mayer's hematoxylin and eosin.

Preparation of the intact heart

The tubular heart of *Triops* is situated just under the dorsal body wall in the thoracic region. After the legs were removed, the body was opened by a longitudinal incision through the ventral body wall. The digestive and genital organs in the thorax were carefully removed to expose the heart. The head and abdomen were also removed by two transverse cuts through the body at either end of the thorax. Thus, the heart was isolated together with the dorsal body wall to which it was still attached. Then, the heart preparation was placed, ventral side up, in the experimental chamber and was fixed to the bottom by pins through the dorsal body wall. The chamber was perfused with aerated physiological saline solution throughout the experiment. Under these conditions, the heart beat regularly for several hours. The saline was based on the ionic composition of *Triops* hemolymph (Horne, 1966), and had the following composition (mM): NaCl, 75; KCl, 5; CaCl₂, 2; MgCl₂, 1; and Tris-HCl (pH 7.4), 5. In some cases, tetrodotoxin (TTX; Sigma) was added to the saline at various concentrations up to 10^{-6} M.

Electrophysiology

The membrane potential of the heart muscle cell was recorded with a conventional glass capillary microelectrode filled with 3 M KCl (resistance; 10–30 M Ω). Another microelectrode, for current injection, was also in-

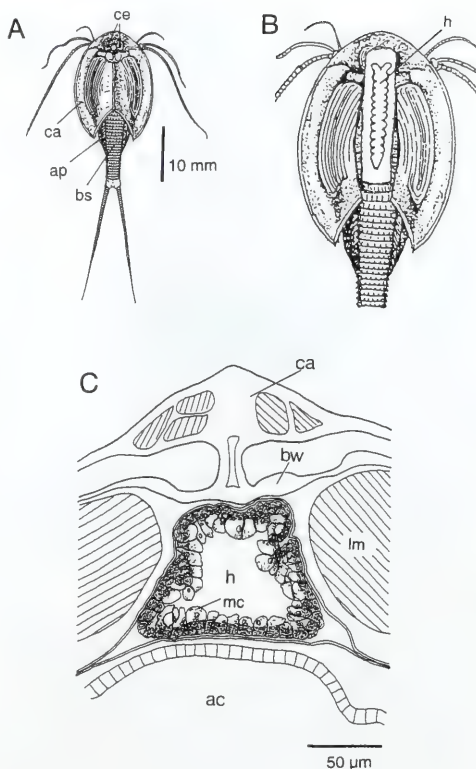


Figure 1. Schematic drawings of the heart of *Triops longicaudatus*. (A) Dorsal view of the adult *Triops*. ap, appendage; bs, body segment; ca, carapace; ce, compound eye. (B) Heart exposed by removal of the carapace and the dorsal body wall. h, heart. (C) Transverse section of the body through the thorax. The heart and surrounding tissues are drawn. ac, alimentary canal; bw, body wall; ca, carapace; h, heart; lm, longitudinal muscle; mc, myocardial cell.

serted into the heart muscle cell, but at a distance from the recording electrode. Contraction of the heart was recorded by placing a high-resistance microelectrode lightly on the dorsal heart wall. The data were stored in an FM magnetic tape recorder and were displayed on a cathode ray oscilloscope and a chart recorder. The experiments were carried out at 20° to 23°C.

Results

Anatomy and histology of the heart

The heart of *Triops*, a long tubular organ, is situated under the dorsal surface of the body (Fig. 1A, B). It

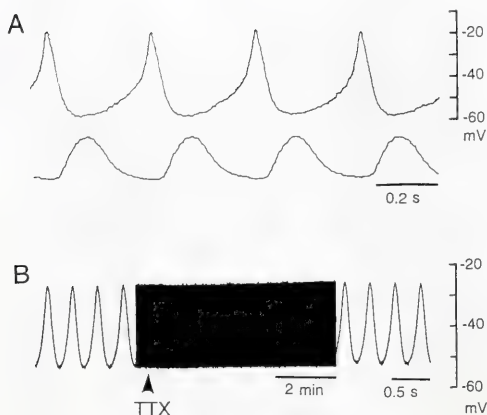


Figure 2. (A) Electrical activity of the heart muscle (upper trace) and contraction of the heart (lower trace) recorded simultaneously. Intracellular membrane potential was recorded from a muscle cell in the anterior region of the heart, and contraction at a site in the central region of the heart. (B) TTX has no effect on the muscle activity. Arrow head: Perfusing solution was changed from normal saline to saline containing TTX (10^{-6} M).

TTX arrests the beat of decapod crustacean hearts by suppressing neuronal spiking in the cardiac ganglion (e.g., Tazaki, 1971). However, the spontaneous slow potentials in the heart muscle of *Triops* were not affected by treatment with TTX at concentrations up to 10^{-6} M (Fig. 2B).

Synchronous activity of the heart muscle cells

Muscle activity was simultaneously recorded from two sites in the heart. Figure 3A shows two examples in which one recording site was near the anterior end (upper traces), and the other near the posterior end of the heart (lower traces). The periodic membrane potential changes were synchronized among the muscle cells within a time difference of less than 50 ms (A1, 16 ms; A2, 12 ms). Thus, the whole heart beat almost simultaneously.

In the case shown in Figure 3A1, the slow potential recorded from the anterior region preceded that recorded from the posterior region. But in the case shown in Figure 3A2, the slow potential recorded from the posterior region preceded that recorded from the anterior region. No local region of the heart had activity that consistently preceded that of the rest of the organ.

extends from the first to the twelfth thoracic segments and is suspended in the pericardial cavity. The heart is 6.4 to 8.6 mm in length, but less than 1 mm in width in the preparations used. The heart tube bifurcates near its anterior end and opens at the head region just under the eyes; its posterior end is closed. The heart has 11 ostia on each side, but no defined walled arteries are present. The heart wall is composed of a single layer of myocardial cells (Fig. 1C). Myofibrils are present in the outer half of the myocardial cell. No nerve cells were found in the heart.

Spontaneous membrane potential changes in the heart muscle

Electrical activity of the heart muscle and contractions of the heart were recorded simultaneously. As shown in Figure 2A, each heartbeat followed a periodic slow depolarizing potential of the heart muscle. The frequency and amplitude of the slow potential were not stable, but changed during measurement in the same preparation. The frequency and amplitude of the slow potentials, measured about 30 min after isolation, were 188.2 ± 32.9 /min (mean $\pm$ SD, $n = 64$) and 17.5 ± 6.3 mV (mean $\pm$ SD, $n = 64$), respectively. No spike potentials appeared on the slow potential. The maximum membrane potential between the slow potentials was 59.2 ± 3.6 mV (mean $\pm$ SD, $n = 56$).

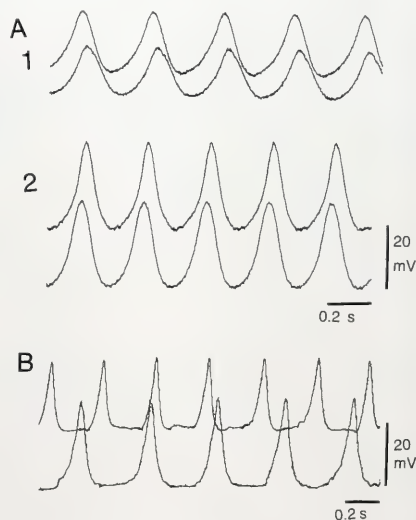


Figure 3. (A) Intracellular muscle activity recorded simultaneously from two sites in the heart: one near the anterior end (upper traces), and one near the posterior end (lower traces). Recordings 1 and 2 are from different preparations. (B) Intracellular muscle activity recorded simultaneously from two separated fragments of the same heart. The heart was cut transversely into four separated fragments. Reckoning from anterior to posterior, the records were from the first (upper trace) and the third (lower trace) fragments.

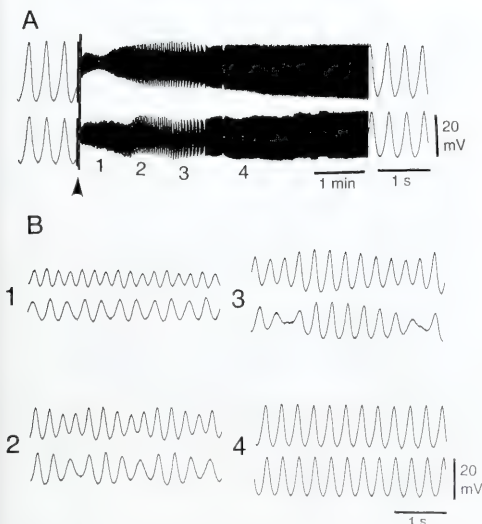


Figure 4. (A) Intracellular muscle activity recorded simultaneously from two sites: near the anterior end (upper trace) and near the posterior end (lower trace) of the heart. Arrow head: A mechanical stimulus (touching) with a small glass rod was applied to the central region of the heart. (B) Records with a faster sweep. The number of each record corresponds to the number in (A).

We next examined muscle activity in cut fragments of the heart. Figure 3B shows an example in which the heart was cut transversely into four separated fragments, and muscle activity was recorded simultaneously from two of them. Activity similar to that found in the intact heart was recorded from the two fragments, each with its own rhythm. Thus, a fragment isolated from any portion of the heart exhibited spontaneous rhythmic activity.

A mechanical stimulus applied to the heart by touching or pressing the heart wall with a small glass rod produced a transient systolic arrest within a localized region near the stimulated site. When the central region of the heart was arrested by the local application of a mechanical stimulus, the rhythmical activities in the anterior and the posterior regions of the heart gradually became synchronized. We therefore examined the time course for achieving synchronization of the muscle activities. The experiment in Figure 4 is exemplary. Before application of the stimulus (Fig. 4A, left portion), the slow potentials recorded from the anterior (upper trace) and posterior (lower trace) regions were synchronized at a frequency of 177/min with a time difference of about 35 ms. Upon stimulation, the muscle cell membranes in both regions depolarized by more than 10 mV, and then recovered

gradually to the control level (Fig. 4A, middle and right portions). After the stimulation (Fig. 4B), the frequency of the slow potential increased conspicuously (270/min) in the anterior region, but was almost unchanged in the posterior region (B1). Subsequently, the frequency and amplitude of the slow potential in both regions changed and the rhythm became irregular (B, 2 and 3); then the rhythm became synchronized as in the control condition (B4).

Electrical coupling among the heart muscle cells

Two electrodes, one for current injection and the other for recording, were inserted into muscle cells at different sites of the heart. In the experiment of Figure 5A, the

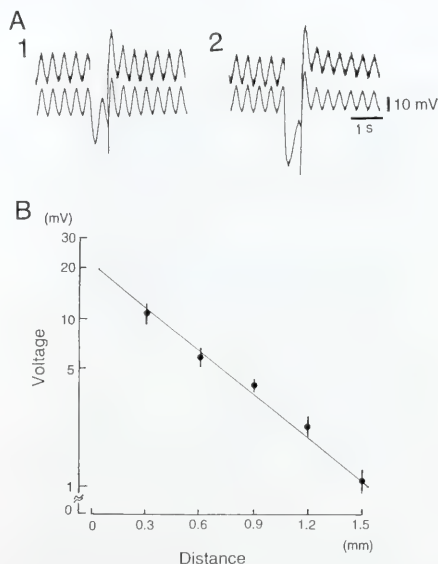


Figure 5. Electrical coupling among the heart muscle cells. (A) Intracellular muscle activity recorded simultaneously from two sites in the heart. Distance between the two electrodes was about 600 μ m. A hyperpolarizing current pulse, 500 ms in duration, was injected into the heart muscle through one of the two electrodes, using a bridge circuit (upper traces). Intensity of the current was 100 nA in A1 and 200 nA in A2. (B) Relationship, in an arrested heart, between the amplitude of the electrotonic potential and the distance between the current and the recording electrodes. A hyperpolarizing current pulse (100 ms, 80 nA) was injected into the heart muscle through the current electrode, and the resultant electrotonic potential was recorded at various distances from the current electrode. The amplitude of each electrotonic potential was plotted on a logarithmic scale against each distance from the current electrode. Mean values ($\pm$ SEM) of 5 measurements in the same heart were plotted.

two electrodes were about 600 μm apart. When a hyperpolarizing current pulse (500 ms, 100 nA) was injected, the membrane of the muscle cell hyperpolarized (A1). The amplitude of the hyperpolarization increased with the intensity of the current injected (A2, 200 nA).

The spread of electrotonic potentials was examined with heart preparations that had stopped beating after perfusion for several hours. The electrotonic potential produced by injecting a hyperpolarizing current pulse (100 ms, 80 nA) was recorded while the distance between the current and recording electrodes, along the ventral longitudinal midline of the heart, was varied. The amplitude of the hyperpolarization decreased with increasing distance between the two electrodes. A semi-logarithmic plot of the amplitude of hyperpolarization against the distance between the current and the recording electrode shows the amplitude decreasing linearly with the distance (Fig. 5B). The length constant (λ) calculated from the relationship was 0.55 ± 0.08 mm (mean $\pm$ SD, $n = 5$).

Effects of current injection on the muscle activity

A brief depolarizing or hyperpolarizing current pulse (50 ms, 50 nA) was injected into the heart muscle at various phases of the rhythmical activity. The inter-peak interval was defined as the time between the peaks of two slow potentials. The phase of the applied current pulse (stimulation phase) was defined as the interval between the peak of the slow potential preceding the pulse and the onset of the pulse; it was taken as a percentage of the preceding inter-peak interval (control inter-peak interval). The resultant phase shift of subsequent beat cycles was measured at the peak of the third slow potential from the applied stimulus; it was taken as a percentage of the control inter-peak interval.

As shown in Figure 6A, a depolarizing pulse applied at 27% produced a phase delay (A1), and a pulse applied at 64% produced a phase advance in the subsequent beat cycles (A2). Figure 6B shows the relationship between the phase shift and the stimulation phase (phase-response curve). The curve was biphasic at a stimulation phase of approximately 40%: a stimulus applied at an earlier phase produced a phase delay (+), and a stimulus applied at a later phase produced a phase advance (-). In contrast, as shown in Figure 7A, a hyperpolarizing pulse applied at 36% produced a phase advance, and one applied at 85% produced a phase delay in the subsequent beat cycles. The phase response curve was biphasic at a stimulation phase of approximately 50%: stimuli applied at an earlier phase produced a phase advance (-), and stimuli applied at a later phase produced a phase delay (+) (Fig. 7B).

We next examined the effects on the muscle activity of injecting repeated brief current pulses. A brief depolarizing or hyperpolarizing current pulse (50 ms, 50 nA) was

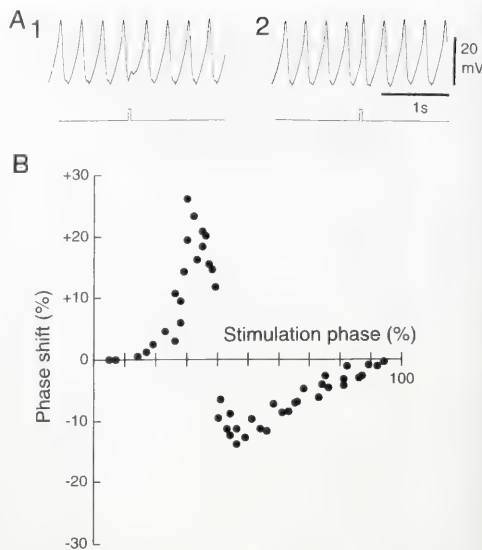


Figure 6. Effects on the muscle activity of injecting a brief depolarizing current pulse. (A) Each record shows the intracellular activity of the heart muscle (upper trace) and a monitor of the stimulus (lower trace). The recording electrode was always inserted near the anterior end of the heart, and the current electrode near the posterior end. A brief depolarizing current pulse (50 ms, 50 nA) was applied at various phases (stimulation phase) of the inter-peak interval. See text for definitions of the stimulation phase and the inter-peak interval. Stimulation phases were 27% (1) and 64% (2). (B) Relationship between phase shift and stimulation phase (phase-response curve). Fifty brief depolarizing pulses were applied at various stimulation phases. Each phase delay (+) or phase advance (-) was plotted against each stimulation phase.

injected into the heart muscle at various frequencies. For example (Fig. 8), muscle activity at a control frequency of 150/min could be entrained to a frequency 17% lower (A1), or 13% higher (A2), by application of repeated brief depolarizing pulses. Similarly, muscle activity could be entrained to a frequency 19% lower (B1), or 13% higher (B2), than the control by application of repeated brief hyperpolarizing pulses.

We examined effects of injecting, for 10 s, depolarizing or hyperpolarizing DC current of various intensities into the heart muscle. Figure 9 shows an example. The frequency of slow potentials increased slightly (A, +2.1%) when depolarizing DC current (10 nA) was applied. The effect increased (B, +6.8%; C, +9.6%) with the intensity of applied current (B, 20 nA; C, 30 nA). By contrast, hyperpolarizing DC current (D, 40 nA) decreased (-13.0%) the frequency of the slow potentials, and the decrease was larger (E, -17.1%) as the intensity of the

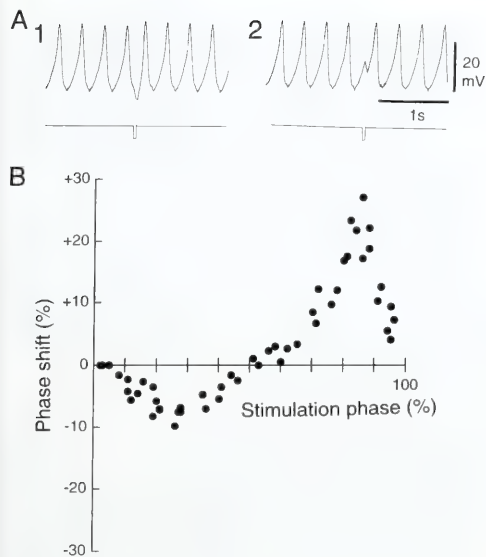


Figure 7. Effects on the muscle activity of injecting a brief hyperpolarizing current pulse. (A) Each record shows the intracellular activity of the heart muscle (upper trace) and a stimulus monitor (lower trace). The recording electrode was inserted near the anterior end of the heart, and the current electrode near the posterior end. A brief hyperpolarizing current pulse (50 ms, 50 nA) was applied at various phases (stimulation phase) of the inter-peak interval. See text for definitions of the stimulation phase and the inter-peak interval. Stimulation phases were 36% (1) and 85% (2). (B) Relationship between phase shift and stimulation phase (phase-response curve). Fifty brief hyperpolarizing pulses were applied at various stimulation phases. Each phase delay (+) or phase advance (−) was plotted against each stimulation phase. From the same preparation as in Fig. 6.

applied current increased (E, 50 nA). Hyperpolarizing DC current of high intensity (F, 60 nA) reduced the amplitude of the slow potential markedly, causing it to disappear. Figure 10 shows the relationship between the frequency change of slow potential and the intensity of current applied. The relationship was almost linear, but hyperpolarization with a DC current more intense than a critical value (50 to 120 nA in 8 preparations) suppressed the slow potentials, and arrested the heart.

The effects of injecting current at various sites on the heart were examined. In Figure 11, for example, the muscle activity was recorded simultaneously near the anterior (upper traces) and the posterior ends (lower traces) of the heart. Muscle activity was suppressed completely by the injection of a hyperpolarizing DC current (5 s, 100 nA) through the anterior electrode (A) or through the posterior electrode (B). Thus, regardless of the site of current injection

in the heart, the same effects on the muscle activity were induced.

Discussion

Branchiopod hearts lack a cardiac ganglion

The heart of *Triops longicaudatus* consists of a single layer of myocardial cells. Myofibrils are found in the outer half of these cells (Fig. 1C). Electron microscopic observations on the heart of another tadpole shrimp, *Lepidulus arcticus*, showed that the heart consists of a single layer of strongly polarized myocardial cells, each with their myofibrillar part facing the epicardium (Tjønneland, *et al.*, 1980). The myofibrils cross each other in the cell and form a contractile network. Tjønneland *et al.* also found intercalated discs between adjacent myocardial cells in the region of the myofibrils.

No nerve cells were found in the heart of *Triops* by

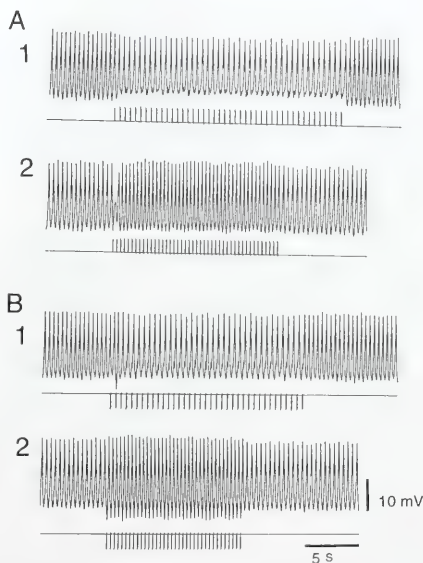


Figure 8. Effects on muscle activity of injecting repeated brief current pulses. Intracellular activity of the heart muscle (upper trace) and a stimulus monitor (lower trace) are shown in each record. The recording electrode was inserted near the anterior end of the heart, and the current electrode near the posterior end. (A) A brief depolarizing current pulse (50 ms, 100 nA) was repeatedly applied at frequencies of 124/min in A1, and 169/min in A2. Frequency of the muscle activity under non-stimulated conditions (control) was 150/min. (B) A brief hyperpolarizing current pulse (50 ms, 100 nA) was repeatedly applied at frequencies of 122/min in B1, and at 169/min in B2. Control frequency of the muscle activity was 155/min. From the same preparation as in Figs. 6 and 7.

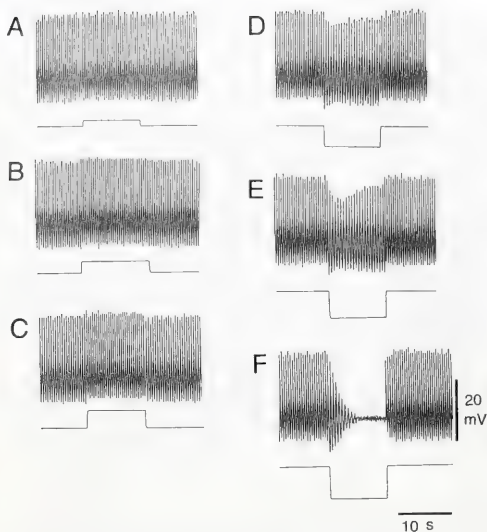


Figure 9. Effects on myocardial activity of injecting DC current. The recording electrode was inserted near the anterior end of the heart, and the current electrode near the posterior end. In each record, intracellular activity of the heart muscle (upper trace) and monitor of the current applied (lower trace) are shown. Depolarizing (+) or hyperpolarizing (−) DC current was applied for 10 s. Intensity of the current (nA) was +10 (A), +20 (B), +30 (C), −40 (D), −50 (E), and −60 (F).

vital staining with methylene blue or by microscopic observation of serial sections of the heart. Ingle (cited by Prosser, 1942) also used various nerve-staining methods, but failed to find nerve cells in the heart of *Daphnia*. Furthermore, although electron microscopic methods have been used to study the hearts of several branchiopods—*Daphnia* (Stein *et al.*, 1966), *Lepidurus* (Tjønneland *et al.*, 1980), *Branchinecta*, *Artemia*, *Branchipus*, and *Streptocephalus* (Økland *et al.*, 1982)—no neural elements have ever been reported. It is unlikely that the heart of branchiopods has a cardiac ganglion.

Myogenic activity

The heartbeat of *Triops* is associated with periodic slow depolarizing potentials in the myocardium (Fig. 2A). This activity was not affected by application of TTX (Fig. 2B). In the neurogenic heart of crustaceans, the heart muscle has no endogenous rhythmicity and is driven by a cardiac ganglion (reviewed by Maynard, 1960; Prosser, 1973). The heart of these animals stops beating if the cardiac ganglion is removed (reviewed by Krijgsman, 1952) or if TTX is applied, suppressing neuronal spiking in the

cardiac ganglion (Anderson and Cooke, 1971). In the myogenic heart of embryos and early juveniles of the isopod *Ligia*, the muscle activity is not affected by application of TTX, except for the TTX-sensitive spike on the slow potential (Yamagishi, 1996; Yamagishi and Hirose, 1997). The ineffectiveness of TTX on the *Triops* heart supports the idea that the rhythmicity of this myocardium is generated endogenously.

The electrical activity of the *Triops* heart consists of only slow potentials, and the amplitude of the slow potential changes gradually during measurement. But no spike potentials ever appeared, even after termination of a hyperpolarizing DC current injected into the heart muscle (e.g., Fig. 8). The heart muscle of *Triops*, generating membrane potential oscillation, may lack the ability to generate all-or-none membrane potentials.

Characteristics of the heart as a single muscle oscillator

No localized regions of the *Triops* heart consistently generated potentials that preceded those of other regions (Fig. 3A). Moreover, any cut fragment of the heart exhibited spontaneous muscle activity (Fig. 3B). These results suggest that the heart of *Triops* has diffuse myogenicity, and that individual muscle cells are inherently oscillatory. When the central region of the heart was arrested by a mechanical stimulus, the anterior and posterior regions

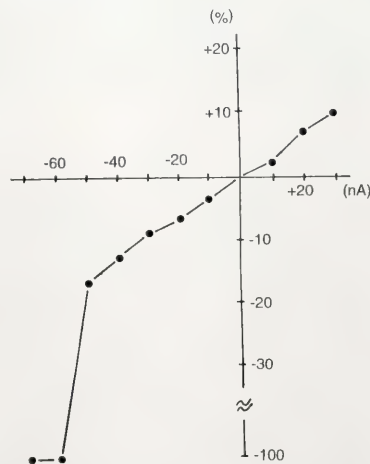


Figure 10. Relationship between the change of frequency of the muscle activity and the intensity of the current applied. Each percentage change in the slow potential frequency of the muscle activity was plotted against the intensity (nA) of the depolarizing (+) or hyperpolarizing (−) current applied. From the same preparation as in Fig. 9.

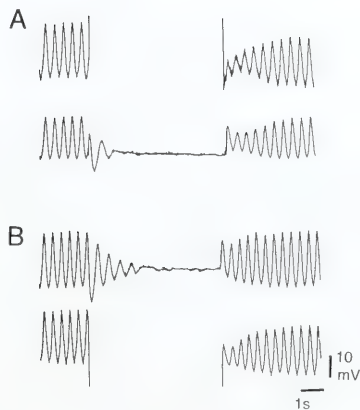


Figure 11. Effects on myocardial activity of current injection at two sites of the heart. Intracellular activity of the heart muscle was recorded simultaneously at two sites in the heart: one near the anterior end (upper traces), and one near the posterior end (lower traces). (A) Hyperpolarizing DC current (100 nA) was injected for 5 s through the electrode inserted at the anterior region, using a bridge circuit. (B) Hyperpolarizing DC current (100 nA) was injected for 5 s through the electrode inserted at the posterior region, using a bridge circuit.

beat independently with their own rhythms; subsequently their rhythms were gradually resynchronized (Fig. 4). This suggests that the heart muscle cells synchronize their activities through interaction. The high sensitivity of the *Triops* heart to mechanical stimuli suggests some stretch-mediated contribution to the synchronization of the muscle cells. However, spontaneous activity was rapidly synchronized (Fig. 3A), and the heart muscle cells appear to be electrically coupled (Fig. 5).

We therefore conclude that the heart muscle cells are synchronized through electrical interactions, as in the myogenic hearts of other invertebrate species (reviewed by Ebara, 1993). The heart of *Triops* can be said to have a diffuse myogenic pacemaker with the properties of a functional syncytium. These characteristics of the heart are very similar to those of the myogenic hearts of embryos and early juveniles of the isopod *Ligia* (Yamagishi and Hirose, 1997) and of most molluscs (reviewed by Hill and Welsh, 1966; Irisawa, 1978; Jones, 1983).

The rhythm of the muscle activity could be phase-shifted by a brief current pulse (Figs. 6 and 7) and could be entrained to the frequency of repeated, brief current pulses injected into the muscle cell (Fig. 8). In addition, the frequency of the muscle activity changed linearly with the intensity of the injected DC current, and the muscle activity was suppressed completely during the injection of strong hyperpolarizing DC current (Figs. 9 and 10).

These responses to external inputs are characteristic of endogenous oscillators in general (e.g., Pavlidis, 1973). Moreover, these effects on muscle activity could be induced by current injection at any site in the heart (Fig. 11). Thus, the heart of *Triops* can be regarded as a single muscle oscillator.

Diversity in the pacemaker mechanism of crustacean hearts

Many investigations on the heartbeat pacemaker mechanism of crustaceans, mainly of decapods, have led to the generalization that crustacean hearts are neurogenic, with the cardiac ganglion acting as the dominant pacemaker (reviewed by Krijgsman, 1952; Maynard, 1960; Prosser, 1973). Recently, however, Yamagishi and Hirose (1997) showed that the cardiac pacemaker in the isopod *Ligia exotica* is transferred from the heart muscle to the cardiac ganglion during juvenile development. Now we have shown that the heartbeat of the branchiopod *Triops longicaudatus* is myogenic, and that the heart muscle acts as a diffuse pacemaker. Clearly, the heartbeat pacemaker mechanism in crustaceans is more diverse than previously thought.

Acknowledgments

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Polyphyly of “Sclerosponges” (Porifera, Demospongiae) Supported by 28S Ribosomal Sequences

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Abstract. To test the competing hypotheses of polyphyly and monophyly of “sclerosponges,” sequences from the 5' end of 28S ribosomal RNA were obtained for *Astrosclera willeyana*, *Acanthochaetetes wellsi*, and six other demosponge species. Phylogenetic relationships deduced from parsimony and neighbor-joining analyses suggest that these sclerosponges belong to two different orders of Demospongiae: *Astrosclera willeyana*, being closely related to the Agelasidae, belongs to the Agelasida, *Acanthochaetetes wellsi*, being closely related to the Spirastrellidae, belongs to the Hadromerida. These results contradict the hypothesis that sclerosponges are monophyletic and imply that a massive calcareous skeleton has evolved independently in several lineages of sponges.

Introduction

Recent sponges generally have a skeleton made of spicules that are either siliceous (classes Demospongiae and Hexactinellida) or calcareous (class Calcarea). However, 16 living species build an unusual solid calcareous skeleton, which bears a striking similarity to that of various Cnidaria, in addition to this spicular skeleton. These “coralline sponges” are believed to be the survivors of the stromatoporoids, sphinctozoans, and chaetetids, important ancient reef builders that were highly diversified

during the Paleozoic and Mesozoic eras and that were long thought to be extinct (Hartman and Goreau, 1970; Vacelet, 1983; Wood, 1990).

Since the discovery of these living coralline sponges, they have been classified according to three systems, each reflecting a different belief in the number of times that sponges have invented a massive calcareous skeleton. In the first, all of the coralline sponges are included in the class Ischyrospongiae (Termier and Termier, 1973). In the second, the massive calcareous skeleton is believed to have evolved at least twice, once among coralline sponges with similarities to the Calcarea and once among coralline sponges that more closely resemble the Demospongiae; the latter group is assigned to the class Sclerospongiae (Hartman and Goreau, 1970). This second interpretation has been the most widely used, appearing in many recent treatises on zoology (Parker, 1982; Riedl, 1983) and paleontology (Rigby and Stearn, 1983). A third system (Vacelet, 1979, 1985) reflects the assertion that the massive calcareous skeleton is more plastic and has evolved in several different lineages within the Demospongiae and Calcarea. Under this system, living and, where possible, fossil coralline sponges are classified within the various taxa of Demospongiae and Calcarea with which they share derived characters.

Three coralline sponges are included in this study: *Acanthochaetetes wellsi* Hartman and Goreau, 1975; *Astrosclera willeyana* Lister, 1900; and *Petrobiona mas-siliana* Vacelet and Lévi, 1958. *Acanthochaetetes wellsi* and *Astrosclera willeyana* are of special interest, because

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they are considered to be living representatives of chaetetes and stromatoporoids, two groups of great importance in the fossil record. The affinities of these groups were previously uncertain, but they were most often classified in the Cnidaria (Lecompte, 1956; Fischer, 1970). The separation of the two groups implies an independent derivation of the massive calcareous skeleton. The spicular and cytological characters of both species strongly resemble those found in well-defined families of non-calcified demosponges. The choanocytes of *Acanthochaetetes wellsi* possess a periflagellar sleeve; a central cell at the apople of the choanocyte chambers, as in the Hadromerida; and a spicule complement similar to that of the family Spirastrellidae in the order Hadromerida (Hartman and Goreau, 1975; Vacelet and Garrone, 1985; Reitner and Engeser, 1987; Boury-Esnault *et al.*, 1990). *Astrosclera willeyana* has small choanocyte chambers, flattened choanocytes, verticillate acanthostyles, and chemical affinities with the order Agelasida (Hartman and Goreau, 1970; Vacelet, 1981; Boury-Esnault *et al.*, 1990; Williams and Faulkner, 1996). *Petrobiona massiliana* has morphological affinities with the class Calcarea.

In this work we generate a new, independent data set based on DNA sequences, use it to construct phylogenies, and compare these with morphological ones. Our main objective is then to determine which of the three hypotheses are consistent with the molecular phylogeny.

Materials and Methods

Material: selection and preservation

The species analysed and their sites of collection are listed in Table I. Some demosponge species were selected as representatives of the various taxa supposedly related to *Astrosclera* and *Acanthochaetetes*. Other species with various levels of distance from the in-group were chosen: these include representatives of other demosponge sub-classes—one Ceractinomorpha species (*Halichondria panicea*) and two Tetractinellida species (*Cinachyrella sp.* and *Discodermia polydiscus*)—and of class Calcarea (*Clathrina cerebrum*), with which *Petrobiona massiliana* has affinities. For further convenience, all demosponge species that are not Tetractinellida are grouped under the collective term "monactinellids." All specimens were either preserved in 70% ethanol or deep-frozen in liquid nitrogen and then kept at -80°C , depending on collecting conditions.

DNA processing

Extraction. The total genomic DNA extraction technique was modified from the Simple Fool's Guide to PCR (Palumbi *et al.*, 1991). Less than 0.5 g of tissue

was crushed in a sterile mortar after total dehydration (overnight air-dry at $+4^{\circ}\text{C}$ or speed vac) for the alcohol-preserved samples and in liquid nitrogen for the frozen samples. The powder was gently mixed for a few minutes with 500 μl of lysis buffer (Palumbi *et al.*, 1991). Spicules and cellular remains were then removed by centrifugation for 2 min at 13,000 rpm. Digested tissue was purified successively in phenol, phenol-chloroform-isoamylalcohol, and chloroform-isoamylalcohol extractions. Nucleic acids were precipitated with ammonium acetate-isopropanol, followed by a 70% ethanol wash. Total DNA was resuspended in sterile distilled water and its concentration determined by optical density at 260 nm.

Polymerase chain reaction. Two overlapping fragments of the ribosomal RNA gene were amplified using a universal primer and a sponge-specific primer. Primers used were as follows (specificity, orientation, and position of primers in the aligned sequences of Figure 1 follow each sequence): ITS3 5'-GTCGATGAAGAACGCAGC-3', universal, forward, external 5'; Ep1b' 5'-GTGGCCGGGAGAGGCAGC-3', part of Demospongiae not Tetractinellida, forward, 257–274; Ep2 5'-CTTYGACGTGCC-TTCCAGGT-3', Demospongiae, reverse, 303–323; D2 5'-TCCGTGTTTCAAGACGGG-3', universal, reverse, external 3'.

The fragment "ITS3-Ep2" contains a part of the 5.8S rRNA gene, the ITS2, the C1 domain and half of the D1 domain of the 28S rRNA gene; the "Ep1b'-D2" fragment contains the other half of the D1 domain, the C2 and the D2 domains of the 28S rRNA gene.

A 50 μl double-stranded PCR reaction mix contains 0.3 μg template DNA, 2.5 μl DMSO, 0.165 mM each dNTP, 30 pmol each probe, 1.5 U Taq DNA polymerase (Bioprobe). This reaction mix was overlaid with mineral oil and placed in a Trio-thermoblock thermocycler (Biometra). Cycling conditions are variable for the annealing temperature (Ta): respectively 60°C and 63°C for ITS3-Ep2 and Ep1b'-D2 primer pair. The first cycle is 4 min at 94°C , 2 min at Ta, and 2 min at 72°C ; this is followed by 30 cycles each consisting of 1 min at 94°C , 1 min at Ta, and 1 min at 72°C ; the reaction is finished by 4 min at 72°C .

After visualization of 5 μl of the reaction on a 1.5% agarose gel, the remaining 45 μl of PCR product was purified by precipitation with ammonium acetate-isopropanol, followed by a 70% ethanol wash. The pellet was then resuspended in 6 μl of sterile distilled water.

The approximate concentration was evaluated visually by electrophoresis of 1 μl of the purified PCR product in a 1.5% agarose gel, and comparison to 1.5 μl of the DNA molecular weight marker VI (Boehringer Mannheim).

Cloning and sequencing. Each PCR fragment was cloned into PCR-Script SK(+) cloning vector (PCR-

Table 1

Sponge species sequenced for analysis of phylogenetic relationships among sclerospoges

Classification	Species	Collection locality
DEMOSPONGIAE		
Tetractinellida		
Tetillidae	<i>Cinachyrella</i> sp.*	New Caledonia
Theonellidae	<i>Discodermia polydiscus</i> Bocage, 1870*	Mediterranean sea, 3PP cave, La Ciotat
"monactinellids"		
Axinellidae	<i>Axmella damicornis</i> (Esper, 1794)*	Mediterranean sea, La Ciotat
Agelasidae	<i>Agelas oroides</i> (Schmidt, 1864)**	Mediterranean sea, La Ciotat
Astroscleridae	<i>Astrosclera willeyana</i> Lister, 1900	New Caledonia 1992
	<i>Astrosclera2 willeyana</i> Lister, 1900	New Caledonia 1994
Clionidae	<i>Cliona viridis</i> (Schmidt, 1862)*	Mediterranean sea, La Vesse
Spirastrellidae	<i>Spirastrella cf. coccinea</i> (Duchassaing & Michelotti, 1874)	Panama, Atlantic coast San Blas Island
Acanthochaetidae	<i>Acanthochaetetes wellsi</i> Hartman & Goreau, 1975	New Caledonia 1992
Halichondriidae	<i>Halichondria panicea</i> (Pallas, 1766)*	South West Channel, Aber Wrac'h
CALCAREA		
Clathrinidae	<i>Clathrina cerebrum</i> (Haeckel, 1872)*	Mediterranean sea, La Vesse
Petrobionidae	<i>Petrobionia massiliana</i> Vacelet & Lévi, 1958**	Mediterranean sea, Anse des Cuivres

* Sequences from Chombard *et al.* (In press).** Sequences from Lafay *et al.* (1992).

Script SK(+) cloning kit, Stratagene) and sequenced with the T7 Sequencing kit (Pharmacia Biotech) using [33P]-dATP and adding DMSO in the annealing reaction. The internal probe C2' is used to obtain the middle of the "Ep1b'-D2'" fragments, in addition to the vector probes Ks and T3 (C2' 5'-GAAAAGAACTTGRARAG-AGAGT-3', universal specificity, forward orientation, position 483-505 on the aligned sequences of Figure 1).

Each PCR product was sequenced from a minimum of two clones; when contradictions in the sequences of several clones could not be resolved, the corresponding positions were coded according to the UPIAC code. The two strands were sequenced for the main part of the sequence length, with special attention to the D2 domain where strong secondary structures of the molecule cause compressions in the sequence migration. From the two overlapping PCR products, the final sequence was 1104 bp to 1197 bp in length, depending on the species. This fragment corresponds to the 3' extremity of the 5.8S rRNA (about 108 bp), the Internal Transcribe Spacer ITS2 (between 167 bp and 224 bp), and the four first domains of the 5' extremity of the 28S rRNA: C1, D1, C2, and D2 (between 816 and 866 bp).

Sequence management and alignment

The MUST package (Philippe, 1993) was used to manage sequences, including registration (with ENTRYSEQ program), alignment (with ED), construction of distance

matrices (with NET or from NJ trees), distance calculations and construction of trees with the neighbor joining algorithm (with NJ), matrix comparison (with COMP-MAT), and calculation of bootstrap proportions from neighbor joining trees (with NJBOOT). Wherever likely secondary structures were detected, sequences were aligned according to supposed conservation of helices.

PAUP, version 3.1.1 (Swofford, 1991), was also used for construction of trees and calculation of bootstrap proportions, discussed below. In bootstrap calculations, non-majority nodes were compared in order to explore the robustness of alternative topologies.

The final alignment presented in Figure 1 was obtained by eye using the editor of MUST (ED). The ITS2 (not presented in Fig. 1) and part of the 5' extremity of the D2 domain (corresponding to positions 575-640, Fig. 1) are very divergent and cannot be aligned in all our samples, thus these regions were not used in the sequence analysis.

Results

Because previously published sequences of 28S rRNA (Lafay *et al.*, 1992) are shorter than ours, two successive analyses were made. The first grouped all species and corresponds to the length published by Lafay *et al.* (Table I); in the second, *Clathrina*, *Petrobionia*, and *Agelas* were removed so that we could use our total alignable length.

The first analysis included 12 species and 374 bp of

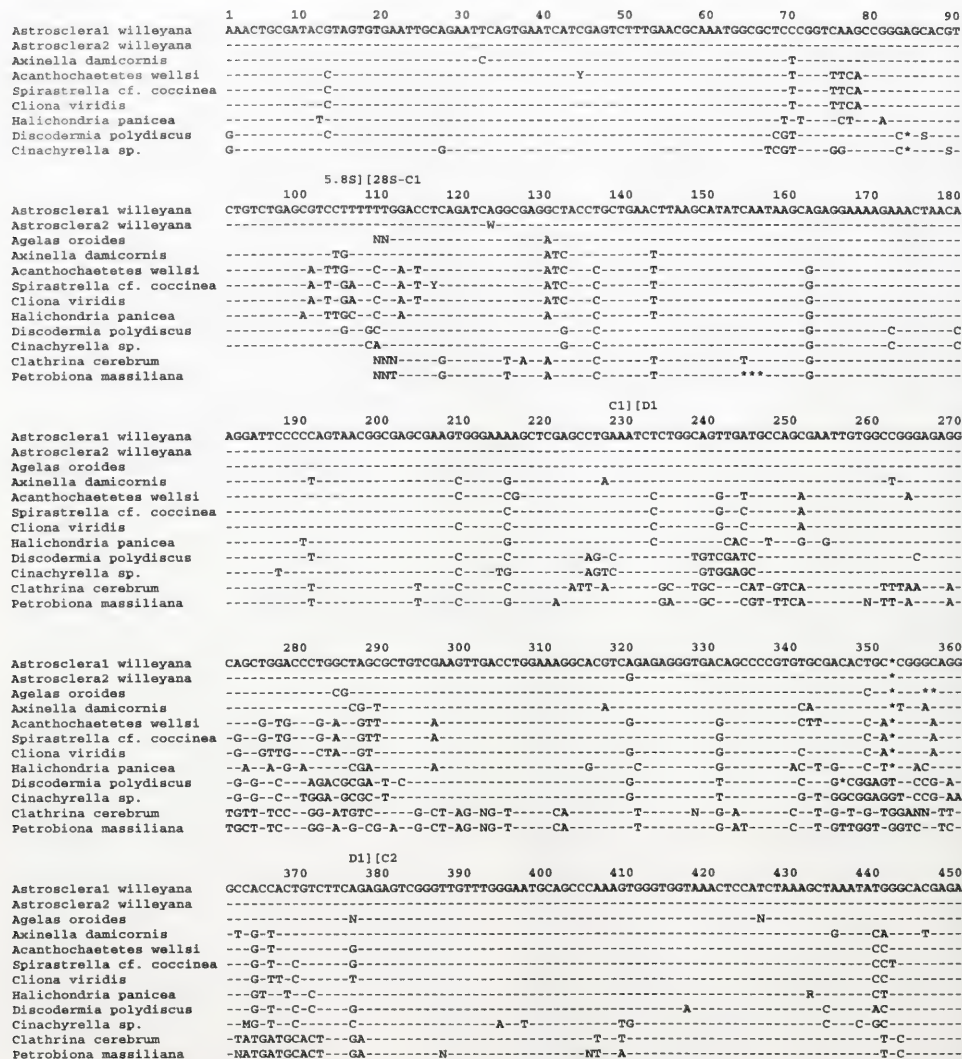


Figure 1. Aligned sequences. Only nucleotides that differ from those of *Astrocleral* are indicated (identities are noted by hyphens and deletions by stars). Boundaries between 5.8S gene and 28S gene are indicated over the sequences, as boundaries between domains of the 28S gene. Crosses over sequences indicate the nonalignable part of the D2 domain, which is not used for phylogenetic analysis.

sequence, 145 of which are variable and 106 informative for parsimony. As shown in Figure 1, these sequences include the C1, D1, and part of the C2 domains of the 28S

rRNA gene. Saturation was tested using COMP-MAT of MUST. Global saturation is not detected, observed distances and number of steps inferred by PAUP between

	460	470	480	490	500	510	520	530	540
Astrocleral willeyana	CCGATAGCAAA	CAAGTACCGT	GAGGGAAGGT	GAAAGTACT	TTGTAAGAGAG	AGTCAAAAGAC	CCCGAAACCG	TAGGAGGGA	AGCGAA
Astrocleral2 willeyana	-----G-----	-----C-----	-----N-----						
Agelas oroides	-----G-----								
Axinella damicornis	-----G-----	-----A-----						-----A-----	
Acanthochaetetes wellsi	-----G-----	-----A-----						-----A-----	
Spirastrella cf. coccinea	-----G-----	-----A-----						-----A-----	
Cliona viridis	-----G-----	-----A-----						-----A-----	
Halichondria panicea	-----G-----	-----A-----						-----A-----	
Discodermia polydiscus	-----G-----	-----A-----						-----A-----	
Cinachyrella sp.	-----G-----	-----A-----						-----A-----	
Halichondria panicea	-----G-----	-----A-----						-----A-----	
Discodermia polydiscus	-----G-----	-----A-----						-----A-----	
Cinachyrella sp.	-----G-----	-----A-----						-----A-----	
Petrobia massiliana	-----G-----	-----A-----						-----A-----	
	550	560	570	580	590	600	610	620	630
Astrocleral willeyana	TGCAGCGAAA	GTGGTCTCT	GCTCAGGCT	CAGGAG**	TTGTTGGCGT	GCAGTGCT	GGGATGCC	AGACCGCG	TAGGGTGC
Astrocleral2 willeyana	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Axinella damicornis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Acanthochaetetes wellsi	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Spirastrella cf. coccinea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cliona viridis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Halichondria panicea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Discodermia polydiscus	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cinachyrella sp.	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
	640	650	660	670	680	690	700	710	720
Astrocleral willeyana	ACGGCTGT**	CGACTGCTT	GCTCAGGCT	CAGGAG**	CCGGCCAA	CGGCAGTTA	CCCTCGGT	CTAAAGGG	GTGTGGGA
Astrocleral2 willeyana	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Axinella damicornis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Acanthochaetetes wellsi	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Spirastrella cf. coccinea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cliona viridis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Halichondria panicea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Discodermia polydiscus	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cinachyrella sp.	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
	730	740	750	760	770	780	790	800	810
Astrocleral willeyana	TTCTCGGTT	*****TAC	CGGGAAGAA	CTTACAGC	CGGCAAC	CGTGCA*	GTCGGGAG	TGACTGAG	GACTGCTG
Astrocleral2 willeyana	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Axinella damicornis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Acanthochaetetes wellsi	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Spirastrella cf. coccinea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cliona viridis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Halichondria panicea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Discodermia polydiscus	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cinachyrella sp.	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
	820	830	840	850	860	870	880	890	900
Astrocleral willeyana	CACCCGCG	AGTACAGG	TCCCT*****	AGGGGGCC	*GGGTCCCT	CTGTGTGT	GGGCAAC	CGCCAGG	*ACTGCAT
Astrocleral2 willeyana	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Axinella damicornis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Acanthochaetetes wellsi	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Spirastrella cf. coccinea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cliona viridis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Halichondria panicea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Discodermia polydiscus	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cinachyrella sp.	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
	910	920	930	940	950	960	970	980	
Astrocleral willeyana	CGGACGG*	ATGTGTGT	CTCAGTGG	GAGGTCCG	*CAGGTCT	TGTGTGT	GTATGTCG	*TTGGTAC	CTGTGCTT
Astrocleral2 willeyana	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Axinella damicornis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Acanthochaetetes wellsi	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Spirastrella cf. coccinea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cliona viridis	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Halichondria panicea	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Discodermia polydiscus	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----
Cinachyrella sp.	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----	-----G-----

Figure 1. (Continued)

all the pairs of species in the data set being linearly correlated (CC 0.98, Fig. 2). Three groups of dots are clearly detectable in this saturation analysis. They correspond to

decreasing distances between (1) Calcarea-Demospongiae, (2) Tetractinellida-monactinellids, and (3) monactinellids-monactinellids. The exhaustive search algorithm

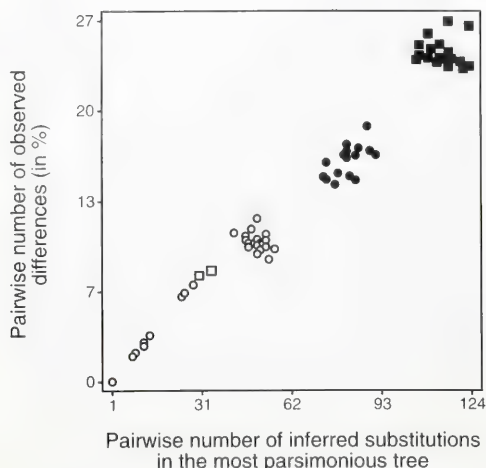


Figure 2. Global saturation curve for 12 species and short-length aligned sequences (374 bp). CC 0.98. White circles are distances between pairs monactinellids-monactinellids. Dark circles are distances between pairs Tetractinellida-monactinellids. Dark squares are distances between pairs Calcareo-Demospongiae. White squares are distances within Tetractinellida and within Calcareo.

of PAUP provided one single shortest tree with 268 steps, a consistency index (CI) of 0.795, a retention index (RI) of 0.767, and a G1 of -1.50. The tree was rooted using the out-group method on both species of Calcareo (*Clathrina cerebrum* and *Petrobionia massiliana*). The resulting single topology is presented in Figure 3. The Branch and Bound search option was used to provide a bootstrap with 1000 replicates in PAUP. The majority-rule consensus

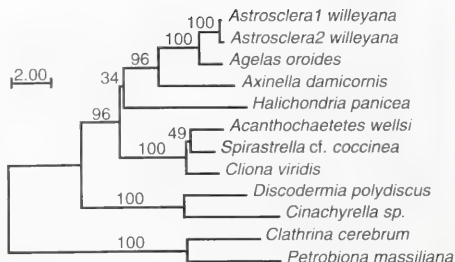


Figure 4. Phylogram obtained with MUST by neighbor-joining analysis on short-length aligned sequences (374 bp) for 12 species. Bootstrap proportions (1000 replicates by NJ analysis) are shown above internal branches.

tree exhibits the same topology as the shortest tree found by exhaustive search (bootstrap proportions [BP] are reported on Fig. 3). The neighbor joining analysis (NJ and NJBOOT in MUST) provided a topology that differs in the location of *Halichondria* and of *Acanthochaetetes*, and in having slightly better bootstrap proportions (Fig. 4).

This first analysis indicates that the Tetractinellida are monophyletic, a conclusion supported by a 100% BP (Chombard *et al.*, in press). This group constitutes the sister group of the other Demospongiae called here "monactinellids." This last group is supported by a 96% BP in distance analysis and an 84% BP in parsimony analysis (Figs. 3–4). All the alternative topologies found by parsimony have less than 5% BP, implying that monactinellids are the monophyletic sister group to the Tetractinellida. For the second analysis of full-length sequences, we are thus able to take the Tetractinellida as an out-group related to the monactinellids. In monactinellids, "sclero-sponges" are polyphyletic. *Acanthochaetetes* is included in a hadromerid clade, in which the monophyly of (*Acanthochaetetes*, *Spirastrella*, *Cliona*) is supported by respectively 100% BP in distance and 99% BP in parsimony analysis (Figs. 3–4). Relationships within this clade are not strongly supported by this first analysis. *Astrosclera* (two individuals) is included in an axinellid clade, in which the monophyly of (*Astrosclera1*, *Astrosclera2*, *Agelas*, *Axinella*) is supported by 96% BP and 83% BP in distance and parsimony analysis respectively. Unlike the hadromerid clade, the axinellid clade has relationships that are well supported—in particular the monophyly of (*Agelas*, *Astrosclera1*, *Astrosclera2*), which is supported by 100% BP and 99% BP in distance and parsimony analysis respectively.

The second analysis was made for 9 species and 914 bp of sequence, 388 of which are variable and 244 informa-

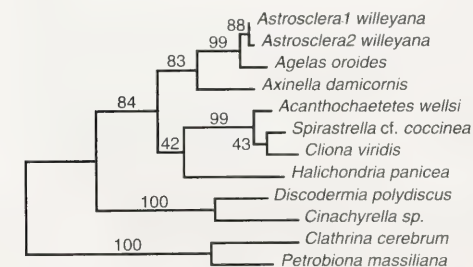


Figure 3. Phylogram obtained with PAUP by exhaustive analysis on short-length aligned sequences (374 bp) for 12 species using ACCTRAN optimization option. Tree length = 268, CI = 0.795, RI = 0.767, and G1 = -1.50. Bootstrap proportions (1000 replicates using Branch and Bound) are shown above internal branches.

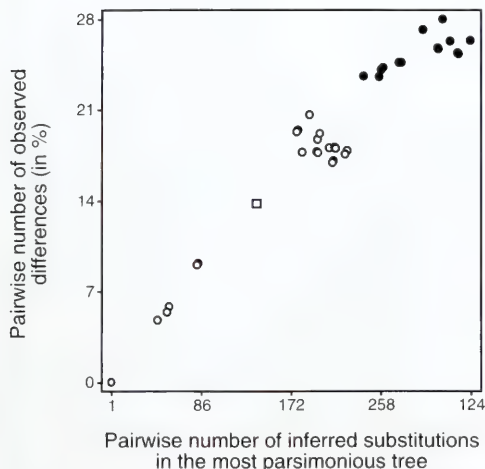


Figure 5. Global saturation curve for 9 species and full-length aligned sequences (914 bp). CC 0.97. White circles are distances between pairs monactinellids-monactinellids. Dark circles are distances between pairs Tetractinellida-monactinellids. White squares are distances within Tetractinellida.

tive for parsimony. No global saturation is evident (CC 0.97, Fig. 5). The exhaustive search algorithm of PAUP provided one single shortest tree with 640 steps, CI = 0.839, RI = 0.783, and G1 = -1.01. The tree was rooted using the out-group method on the tetractinellids (*Cinachyrella* sp. and *Discodermia polydiscus*). The resulting single topology is presented in Figure 6. The Branch and Bound search option was used to provide a bootstrap with 1000 replicates in PAUP. The majority-rule consensus tree exhibits the same topology as the shortest tree found by exhaustive search (BP reported on Fig. 6). The neigh-

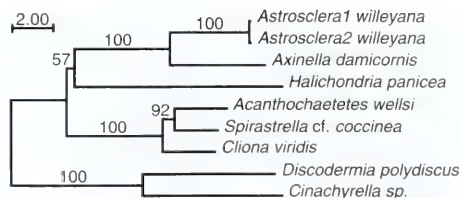


Figure 7. Phylogram obtained with MUST by neighbor-joining analysis on full-length aligned sequences (914 bp) for 9 species. Bootstrap proportions (1000 replicates by NJ analysis) are shown above internal branches.

bor-joining analysis (NJ and NJBOOT in MUST) provided the same topology and similar bootstrap proportions (Fig. 7). This second analysis confirms the first one: the sclerosponges *Astrosclera* and *Acanthochaetetes* belong to two different clades, a hadromerid clade and an axinellid clade. The hadromerid clade (*Cliona*, *Spirastrella*, *Acanthochaetetes*) is supported by 100% BP in both distance and parsimony analysis, and the internal topology is also supported by 92% BP and 88% BP for (*Spirastrella*, *Acanthochaetetes*) in distance and parsimony analysis. The axinellid clade (*Axinella*, *Astrosclera1*, *Astrosclera2*) is supported by 100% BP. The monophyly of the two *Astrosclera* individuals is supported by 100% BP; the individuals came from the same area of New Caledonia and do not represent the two populations, differing by the presence or absence of spicules, that occur respectively in the Indian Ocean and the Central Pacific (Vacelet, 1981; Ayling, 1982).

Discussion

The Ischyrospongiae (Termier and Termier, 1973) hypothesis is falsified by the first analysis. The "coralline" sponge *Petrobionta massiliana* clearly belongs to the class Calcarea, whereas the two other calcified sponges, *Astrosclera* and *Acanthochaetetes*, are undoubtedly part of the Demospongiae. The class Ischyrospongiae is thus polyphyletic, as concluded previously from morphology, and should be abandoned.

Both current analyses demonstrate the polyphyly of the class Sclerospongiae, and it too should be abandoned in classification schemes. Furthermore, the two sclerosponges belong to different monophyletic clades, an axinellid one (*Axinella*, *Agelas*, *Astrosclera*) and a hadromerid one (*Cliona*, *Spirastrella*, *Acanthochaetetes*). Both clades are strongly supported in the two analyses. They are in complete agreement with the affinities indicated by spicule morphology and by cytology (Hartman and Goreau, 1970, 1975; Vacelet, 1981; Vacelet and Garrone,

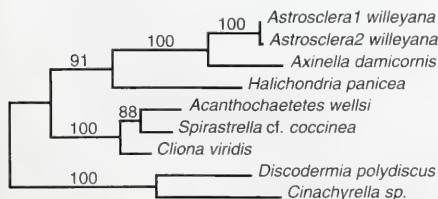


Figure 6. Phylogram obtained with PAUP by exhaustive analysis on full-length aligned sequences (914 bp) for 9 species using ACCTRAN optimization option. Tree length = 640, CI = 0.839, RI = 0.783, and G1 = -1.01. Bootstrap proportions (1000 replicates using Branch and Bound) are shown above internal branches.

1985; Reitner and Engeser, 1987; Boury-Esnault *et al.*, 1990). These results support the interpretation that the capacity to secrete a massive skeleton of calcium carbonate has developed several times during the course of the evolution of the Porifera (Vacelet, 1979, 1983, 1985; Wood *et al.*, 1989). Accordingly, these "coralline" sponges have to be classified in the Demospongiae; *Acanthochaetetes wellsi* in the order Hadromerida; and *Astrosclera willeyana* in the order Agelasida, which is considered by most recent authors as distinct from the order Axinellida, although closely related to it. The creation of a special order—the Tabulospongida—based on the presence of a calcareous skeleton (Hartman and Goreau, 1975) in *Acanthochaetetes wellsi* and its fossil relatives has no strong justification according to the present results.

At a lower taxonomic level, the classification of these sponges as belonging either within existing families to which they are closely related or in distinct families is still subjective. Pending analyses of other related sponges, the decision depends upon individual judgments about the size of the morphological gap needed to separate taxa and about the importance of the calcareous skeleton as a taxonomic character. In the case of *Acanthochaetetes*, we propose to classify the genus in the family Spirastrellidae. Ridley and Dendy, 1886, in view of the spicular and cytological resemblances (periflagellar sleeve, central cell) and the low genetic distance between *Acanthochaetetes* and *Spirastrella* that is indicated by the present work. This hypothesis, which was already proposed by Reitner (1991), avoids the use of the family Acanthochaetidae, which would be monogeneric at least in the Recent fauna. (We reject, however, on the grounds of morphology, Reitner's merging of the genus *Acanthochaetetes* with *Spirastrella*.) In the case of *Astrosclera*, we prefer to maintain the two families Astroscleridae Lister, 1900 (with five genera in the Recent, if merged with Ceratoporellidae), and Agelasidae Verrill, 1907 (with one large genus). The genetic distance between *Astrosclera* and *Agelas*, as estimated by our sequences, is admittedly as low as for *Acanthochaetetes* and *Spirastrella*. However, the Agelasidae and the Astroscleridae differ by an important reproductive character: *Agelas* is oviparous (Liaci and Sciscioli, 1975; Reischwig, 1976), whereas *Astrosclera* is viviparous (Lister, 1900). Furthermore, the structure of the spongin fibers of *Agelas* (De Vos *et al.*, 1991), which are unique among the Demospongiae, is an important difference between *Agelas* and *Astrosclera*.

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Sexual Competition Among Male Blue Crab, *Callinectes sapidus*

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Abstract. Experiments and field data on blue crab, *Callinectes sapidus*, from mid-Chesapeake Bay between 1991 and 1994 were used to test whether large males have advantages over small males in accessing females and in sperm competition. In the field, large males were paired more often, especially with large, more fecund females. However, the variance in the relationship between male and female size in mating pairs was high, suggesting that mating with large females may not be the primary determinant of male reproductive success. Large males had proportionately longer chelipeds, which may provide an advantage in aggressive interactions for females or in struggles to control females. Previous work indicates that sperm competition may occur in blue crabs and that ejaculate size may influence a male's ability to compete during sperm competition. Large males stored more seminal fluid and spermatophores and passed a larger volume of ejaculate to each mate than did small males. Ejaculate volume averaged 47% of a male's stored supply. However, ejaculate volume increased with the duration of copulation but decreased with successive matings, such that males needed about 15 days between matings to pass similar-sized ejaculates to successive mates. Pre-copulatory mate guarding may serve as a time to replenish ejaculate contents, and thus its duration also influences a male's performance in sperm competition.

Introduction

Competition among males for reproductive females is one of the most important forces in the evolution of mating behavior (Andersson, 1994). Males compete for ac-

cess to females when receptive females are numerically, spatially, or temporally limited in relation to males (Trivers, 1972; Emlen and Oring, 1977; Clutton-Brock and Parker, 1992). Males can also compete by means of sperm competition: when females copulate with more than one male and sperm from the different males compete in the female reproductive tract for unfertilized eggs (Parker, 1970; Smith, 1984). Mate guarding is a behavior that males use to compete for access to females as well as in sperm competition (Grafen and Ridley, 1983; Smith, 1984). Pre-copulatory mate guarding often occurs when females are sexually receptive for only a limited period; it ensures that guarding males will have access to a receptive female (Parker, 1974; Grafen and Ridley, 1983). Post-copulatory mate guarding prevents rival males from mating with the inseminated female; therefore, guarding males control access to a female's eggs (Parker, 1970). Mate guarding is widespread among taxa, suggesting that it can enhance mating success in males facing competition from other males (Ridley, 1983; Smith, 1984).

In many species among different taxa, large males have advantages over smaller rivals both in competition for access to females and in sperm competition (Thornhill and Alcock, 1983; Andersson, 1994). Large males often dominate smaller males during aggressive interactions for females (Stein, 1976; Ridley and Thompson, 1979; Ward, 1983; Berrill and Arsenault, 1984), and more easily obtain and physically control females (Berrill and Arsenault, 1982; Carvacho, 1989; Snedden, 1990; Lee and Seed, 1992). As a result, large males can have higher fertilization rates because they more often mate with large, more fecund females (Stein, 1976; Ridley and Thompson, 1985; Forbes *et al.*, 1992; Stevens *et al.*, 1993). The advantages of large male size often result in positive assortative mating; a positive correlation between male and female size in mating pairs (Crespi, 1989). Males may respond to an

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increased risk of sperm competition by guarding for longer durations (Sherman, 1983; Wilber, 1989; Jablonski and Kaczanowski, 1994) or by passing larger ejaculates (Svard and Wiklund, 1989; Gage, 1991; Eady, 1995; Gage and Barnard, 1996), which displace more of a previous inseminator's sperm or prevent dilution by a subsequent inseminator. Compared to small males, large males of some species have greater stores of ejaculate contents (Kwei, 1978; Wilber, 1987; LaMunyon and Eisner, 1993; Pitnick, 1996), pass larger ejaculates even after a number of sequential matings (Markow *et al.*, 1978), and avoid being displaced during mating (Borgia, 1981; Howard and Kluge, 1985; Elwood *et al.*, 1987). Preventing displacement may allow large males to guard or copulate longer, and can result in passing larger ejaculates (McLain, 1980; Svard and Wiklund, 1988).

Male blue crab, *Callinectes sapidus*, exhibit both pre- and post-copulatory mate guarding, suggesting that they may compete both for access to females and in sperm competition. Males may mate several times (although the number of possible matings is undetermined) within a mating season, whereas females mate immediately after their final (pubertal) molt to maturity (Van Engel, 1958; Millikin and Williams, 1980) and usually with a single male (Jivoff, 1997). Thus, theoretically, competition for access to females is expected because at any one time more males than females are available to mate (Clutton-Brock and Parker, 1992). Male blue crab use their chelipeds in both aggressive interactions for females and struggles with females for physical control (Teytaud, 1971; Smith, 1992). In other species, longer chelipeds help large males out-compete smaller males for access to females (Berrill and Arsenault, 1984; Snedden, 1990; Lee and Seed, 1992; Moriyasu and Comeau, 1996; Paul and Paul, 1996), as suggested by mating patterns in the field that show paired males to be larger than unpaired males (Harvey, 1990; Stevens *et al.*, 1993) and reveal some degree of size-assortative mating (Adams *et al.*, 1985; Reid *et al.*, 1994).

Recent experimental and field evidence indicates that sperm competition may occur in blue crab, because some females (about 12%) remate within several days after their final molt, storing both ejaculates in their entirety such that sperm from both males may have equal access to the unfertilized eggs (Jivoff, 1997). As a result, males may enhance their fertilization rate by preventing rivals from mating with the female or by ejaculating greater numbers of sperm in the female relative to that of other males (Parker, 1984). Thus, sperm competition in blue crab can be argued to favor those males that spend a longer time guarding and pass more ejaculate to each female. Indeed, male blue crab increase the duration of post-copulatory mate guarding when the sex ratio is male-biased, and they pass larger

ejaculates in the presence of other males or of previous ejaculates in the female (Jivoff, 1997).

The following paper (1) shows evidence from field data for an advantage of large male size during competition for access to females; (2) experimentally shows how male size, mating history, and copulation duration influence ejaculate volume, which may influence a male's ability to compete during sperm competition; and (3) discusses how competition among males for access to females may interact with sperm competition to influence male mating success.

Materials and Methods

Research was carried out at the Smithsonian Environmental Research Center (SERC) on the Rhode River, a subestuary of Chesapeake Bay, in Maryland (38°51'N, 76°32'W) from mid-June through late September, 1991–1994. All crabs used in experiments were collected in the field. Seines and trawls were sometimes used, but most specimens were taken with a dip net, 2–3 times per week, from the sides of commercial pound nets (length 150–200 m) stretched between vertical posts near the mouth of the Rhode River. Crabs were transported to SERC, measured (see below), separated by sex, maintained in floating field cages in the Rhode River, and fed fish daily until used in experiments.

Variables recorded from field-captured crabs included sex, paired status (pre-copulatory, post-copulatory, copulating, or unpaired), molt stage (see below), sexual maturity (juvenile, pre-pubertal or pre-molt, mature), carapace width (CW; distance, in millimeters, between the tips of the lateral spines), chelae spread (distance, in millimeters, between the tips of the chelipeds when fully extended laterally, 1994 only), and the number and position of missing limbs. Molt stage was determined by examining the propodus on the fifth appendage for evidence of epidermal retraction and color variation and, for recently molted crabs, the relative hardness of the newly formed carapace (Van Engel, 1958). Pre-pubertal females have a triangular, darkened abdomen, whereas adults have a semicircular abdomen. Pre-molt females were designated as follows: early/D₀ (9–10 days pre-molt); early-mid/D₁ (7–8 days pre-molt); mid/D₂ (5–6 days pre-molt); mid-late/D₃ (3–4 days pre-molt); and late/D₄ (1–2 days pre-molt) (Drach, 1939). Males were designated sexually mature according to the criteria of Van Engel (1990): the second pleopods lay within the first pleopods (intromittent organs); the penes were inserted into the second pleopods; and the abdomen easily pulled away from the sternum. I used only mature, intermolt males that possessed both chelipeds and that were missing not more than one walking leg, a condition that does not affect mating behavior or mating success (Smith, 1992). Crabs in experiments

were never held in field cages for more than 1 week and were never reused.

Male blue crabs store spermatophores and seminal fluid in paired vas deferentia (Cronin, 1947; Johnson, 1980). Each vas deferens is connected to an external pleopod, through which seminal fluid and spermatophores are passed to one of the female's two spermathecae (Cronin, 1947; Hartnoll, 1968). No difference was found between the weight of material males store in each vas deferens (paired $t = 0.306$, $df = 10$, $P = 0.766$) or pass through each pleopod (paired $t = 0.276$, $df = 158$, $P = 0.783$) (Jivoff, 1995). Thus, the amount of seminal fluid and spermatophores that males stored in their reproductive tracts and passed to females was weighed using the following protocol. One (randomly assigned) pleopod from each male was removed with dissecting scissors at least 24 h prior to the experiment. After the male copulated once in an experimental pool, each vas deferens was weighed (nearest 0.01 g). The vas deferens with the intact pleopod transferred the normal amount of ejaculate, but the vas deferens lacking a pleopod ("unmated") transferred none. As a result, ejaculate weight was calculated as the difference between the weight of the unmated and mated vas deferentia. The proportion of available material passed was also calculated by dividing the weight of the ejaculate by that of the unmated vas deferens. The surgical procedure proved effective because neither spermatophores nor seminal fluid were lost after pleopod removal, and no difference was found between the calculated weight of the ejaculate passed and the measured weight of the spermathecal contents (paired $t = 0.704$, $df = 42$, $P = 0.485$). The relationships of both seminal stores and ejaculate size to male size were determined with linear regression.

To test whether ejaculate weight served as a measure of the amount of sperm transferred to the female, the relationship between the number of spermatophores within the ejaculate and the weight of the ejaculate was determined. The seminal fluid portion of the ejaculate hardens over time and most of the spermatophores accumulate in a single, large mass at the distal end of the spermathecae, but some are incorporated within the seminal fluid matrix (Johnson, 1980). One spermatheca from each of 29 females was weighed (nearest 0.01 g) and then cut into four equal-sized sections (bisecting longitudinally and again transversely) using dissecting scissors. For a random quarter, the hardened ejaculate was scraped from the inside wall of the spermatheca and the seminal fluid was teased apart under a dissecting microscope to count the spermatophores. Maximum lengths of 25 spermatophores in each ejaculate were measured (to the nearest micrometer). Linear regression was used to determine the relationship between ejaculate weight and both the number of spermatophores and the average length of sperma-

tophores, and between male size and the average length of spermatophores.

The relationship between female size and the weight of the empty spermathecae was determined both as a measure of the sperm storage capacity of females and to estimate the weight of the spermathecal tissue in the total weight of mated spermathecae. Pre-molt females ($n = 27$) were isolated from males, and after their pubertal molt their spermathecae were weighed (nearest 0.01 g). The relationship between average spermathecal weight and female carapace width (pre-molt and adult calculated separately) was determined with linear regression. The weight of the spermathecal tissue was calculated using the appropriate regression equation. In all of the statistical comparisons of the contents of female spermathecae described below, the weight of the spermathecal tissue is removed.

The effect of male and female size on the contents of female spermathecae in the field was estimated using one-way ANOVA to determine how paired male size, adult female size, and female spermathecal contents varied among years. Tukey multiple comparisons tests were used to identify significant differences between years. The spermathecae of the adult females (both paired and unpaired) from each collection date were weighed (nearest 0.01 g). For each year, the relationship between the size of adult females and the total contents of their spermathecae was determined using linear regression. The slopes and elevations of the statistically significant regressions were compared using the t test (Zar, 1984).

Pool experiments

All of the experiments described below were performed in plastic pools (2 m in diameter and 0.3 m deep). Each pool contained about 10 cm of sand and was filled with water from the Rhode River. The pools were constantly aerated and the water was completely changed every 1–2 days. Test salinities matched that of the Rhode River (5–10 ppt), and water temperatures were about 5°C below that of the Rhode River, varying little among pools (22–27°C). Crabs were exposed to the ambient light:dark cycle (14:10). A 0.5-m-high "fence" of hardware cloth was placed around the inside perimeter of each pool and covered with a piece of plywood. The cover protected crabs from terrestrial predators (e.g., raccoons), direct sunlight, and elevated water temperatures. Pools were monitored several times daily for the presence of courtship, copulation, and both types of mate guarding. Crabs in pools were fed two to three frozen, previously crushed, mussels (*Mytilus* sp.) daily.

Male mating history

The effect of male size and number of previous copulations (mating history) on the weight of ejaculate passed

to females during subsequent copulations was determined. Each of six males in each of three size categories (small, <135 mm CW; medium, 135–145 mm CW; and large, >145 mm CW) were mated successively with three virgin females of similar size (90–110 mm CW), randomly assigned among males. The range of male size spanned that of paired males in the field. To ensure that males were previously unmated, they were collected early in the season before any natural matings were observed in the field. Each male was randomly assigned to a test pool, and all males received their first female on the same day. Males were provided successive females within 24 h of their previous copulation; however, differences in female molt stage produced differences in the time between matings (mating interval). Regardless of when males were capable of mating again, mating actually occurred only when the female was ready to molt. After mating, each female's spermathecae were weighed (nearest 0.01 g). The ability of males to replenish their ejaculate contents between matings was estimated by calculating the difference between the weight of ejaculate passed in one mating and that passed in the previous mating. Repeated-measures ANOVA with paired contrasts tested the total weight of both spermathecae from each female as a function of male size category after treatment variances were examined with the Bartlett test. The relationship between ejaculate replenishment and mating interval was determined with linear regression.

Duration of copulation

The effect of male size and copulation duration on the amount of ejaculate passed to virgin females was determined with a two-factor design of male size (small, medium, and large) and copulation duration (1, 2, 4, 8 h.

or uninterrupted, about 12 h). The male size categories were the same as in the mating history experiment (see above). The copulation durations and the start of copulation were designated on the basis of the following observations. The male turns the newly molted female over so his sternum rests on hers; copulation begins after he inserts his pleopods into her vulvae, and may last for 12 h (Van Engel, 1958). Each replicate ($n = 2$) included one mating pair for each treatment combination of male size category and copulation duration. To control the start of each copulation, I provided males with pre-molt females that I predicted would molt within several hours. If a female did not molt on the day she was first introduced, then she was isolated from the male and presented to him the following day, when it was possible to observe the start of copulation. Copulations were interrupted after the designated time by physically separating the mating pair. Each female was sacrificed and her spermathecae were weighed (nearest 0.01 g). Interactive effects of male size and copulation duration on the combined weight of both spermathecae were analyzed with two-way ANOVA after testing for homogeneity of variances with the Bartlett test. A Tukey multiple-comparisons test identified significant differences among treatments. The field and experimental data were analyzed using Systat (SYSTAT, 1992). In the text, means are presented with their standard errors (± 1 SE).

Results

The size of males in the field

The size range of sexually mature males overlapped in each of four years: 1991, 105–179 mm CW; 1992, 95–184 mm CW; 1993, 103–204 mm CW; and 1994, 110–

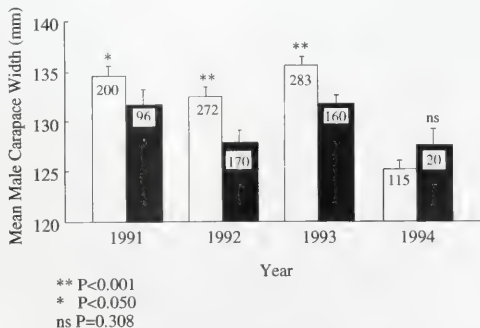


Figure 1. Mean carapace width of pre-copulatory mate-guarding males (open bars) and unpaired mature males, in the intermolt stage only (dark bars), captured in the field during each year of the study. Numbers inside each bar are sample sizes. Vertical bars are 1 standard error.

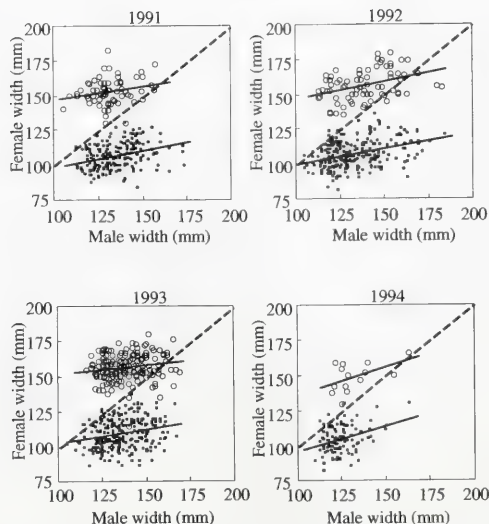


Figure 2. Relationship between guarding-male carapace width and pre-molt (■) and adult (○) female carapace width in mate-guarding pairs captured in the field during each year of the study. The dashed line in each graph indicates where the male and female size are equal. The regression lines for pre-copulatory mate-guarding pairs are as follows: 1991— $Y = 0.24X + 74.44$, $r^2 = 0.099$, $P < 0.001$, $n = 200$; 1992— $Y = 0.22X + 77.11$, $r^2 = 0.123$, $P < 0.001$, $n = 272$; 1993— $Y = 0.20X + 82.31$, $r^2 = 0.064$, $P < 0.001$, $n = 283$; 1994— $Y = 0.36X + 59.46$, $r^2 = 0.104$, $P < 0.001$, $n = 114$. The regression lines for post-copulatory mate-guarding pairs are as follows: 1991— $Y = 0.19X + 127.69$, $r^2 = 0.051$, $P = 0.069$, $n = 66$; 1992— $Y = 0.25X + 121.88$, $r^2 = 0.142$, $P = 0.001$, $n = 73$; 1993— $Y = 0.12X + 139.24$, $r^2 = 0.026$, $P = 0.035$, $n = 174$; 1994— $Y = 0.39X + 96.35$, $r^2 = 0.263$, $P = 0.061$, $n = 14$.

164 mm CW. In three years, the mean CW of paired males was significantly larger than that of unpaired males, but in 1994 there was no significant difference (Fig. 1). In each year, large males were more often paired with large pre-molt females (Fig. 2). Large males were also more often paired with large adult females in post-copulatory mate-guarding pairs in 1992 and 1993 (Fig. 2). Paired males were typically larger than pre-molt females but smaller than their adult female partners. However, in each year, male size explained little (2%–26%) of the variation in the size of paired pre-molt or adult females. A significantly positive allometric relationship ($\beta = 1.148$, $P < 0.001$) occurred between male CW and the distance between the tips of male chelipeds when fully extended laterally (chela spread), indicating that large males have proportionately longer chelipeds than small males (Fig. 3).

The size of males and the weight of ejaculates

The number of spermatophores present in the ejaculate increased significantly with ejaculate weight ($Y = 0.613X + 2.49$, $r^2 = 0.332$, $n = 29$, $P = 0.001$) (Fig. 4); however, no relationship was found between male CW and sperma-

tophore size ($n = 29$, $P = 0.446$) or between ejaculate weight and spermatophore size ($n = 29$, $P = 0.592$). The amount of seminal fluid and spermatophores stored in the vas deferens both before ($Y = 0.04X - 2.40$, $r^2 = 0.263$, $n = 335$, $P < 0.001$) and after ($Y = 0.03X - 1.92$, $r^2 = 0.202$, $n = 335$, $P < 0.001$) one mating increased with male size (Fig. 5). Furthermore, the size-related increase in the weight of seminal products stored before mating was significantly larger than that after mating ($t = 3.12$, $df = 669$, $P < 0.002$), indicating that large males pass more ejaculate than do small males. Weights of female spermathecae contents also indicate that the weight of ejaculate stored by females increased with the size of their mate ($Y = 0.015X - 0.484$, $r^2 = 0.096$, $n = 334$, $P < 0.001$). On average, males passed 46.9% (± 0.008 SE) of their available ejaculate. The relationships between adult female size and the weight of ejaculate received ($n = 212$, $P = 0.172$), and the proportion of ejaculate received ($n = 212$, $P = 0.10$) were not significant.

Contents of female spermathecae in the field

The weight of empty spermathecae increased with size of pre-molt females (Fig. 6). As compared with small

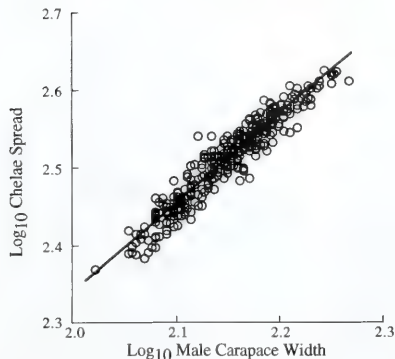


Figure 3. Relationship between $\log_{10}$ carapace width and $\log_{10}$ chelae spread among adult male blue crabs. Chelae spread is the distance (in millimeters) between the tips of the chelae when they are extended laterally to 180° from the anterior. The regression line is described by the following equation: $Y = 0.044X + 1.148$, $n = 358$, $r^2 = 0.922$, $P < 0.001$. The slope indicates significant positive allometry $F_{1,356} = 69.90$, $P < 0.001$.

females, large females carried more ejaculate in both spermathecae in 1994 ($Y = 0.003X + 0.043$, $r^2 = 0.052$, $n = 185$, $P = 0.002$), and 1993 ($Y = 0.001X + 0.453$, $r^2 = 0.011$, $n = 389$, $P = 0.04$), but not in 1992 ($n = 171$, $P = 0.136$). The slopes of the regressions in 1994 and 1993 were not significantly different ($t = 1.8$, $df = 571$, $P > 0.05$); however, in 1993 females had significantly more material stored in their spermathecae than they did in 1994 ($t = 9.4$, $df = 572$, $P < 0.001$). There were significant differences in the spermathecal contents ($F_{2,742} = 53.24$, $P < 0.001$), paired male size ($F_{2,968} = 36.33$, $P < 0.001$), and adult female size ($F_{2,744} = 11.72$, $P < 0.001$) among years (Fig. 7). Furthermore, the pattern of

the differences was consistent among years; the weight of female spermathecal contents was greatest when paired male size and adult female size were the largest (Fig. 7).

Male mating history

Male size ($F_{2,15} = 8.96$, $P = 0.003$) and number of previous mates ($F_{2,30} = 5.19$, $P = 0.012$) had a significant effect on the total ejaculate passed to females. Large males passed significantly larger ejaculates to their first ($F_{1,10} = 10.36$, $P = 0.009$), second ($F_{1,10} = 34.29$, $P = 0.0002$), and third females ($F_{1,10} = 5.80$, $P = 0.036$) than did small males (Fig. 8). Large males passed significantly

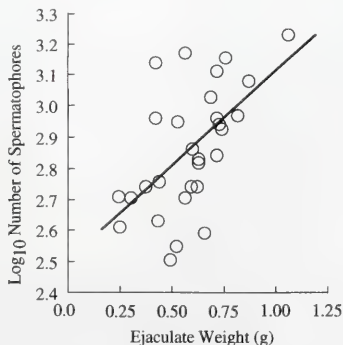


Figure 4. Relationship between ejaculate weight and the $\log_{10}$ number of spermatophores contained in the ejaculate. The regression line is described by the following equation: $Y = 0.613X + 2.49$, $r^2 = 0.332$, $P = 0.001$, $n = 29$.

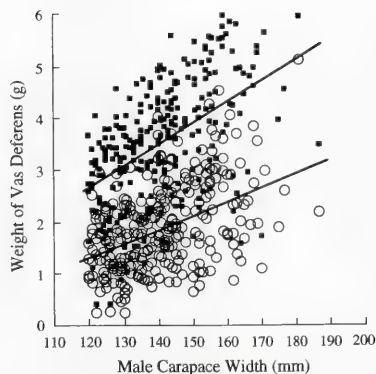


Figure 5. Relationship between male carapace width and the weight of ejaculate stored in the vas deferens before (■) and after (○) mating. Males had one pleopod removed before mating. Ejaculate stored before mating was the weight of the unmated (full) vas deferens. Ejaculate stored after mating was the weight of the mated (spent) vas deferens. The equations for the regression lines are as follows: $Y = 0.04X - 2.40$, $r^2 = 0.263$, $P < 0.001$, $n = 335$ (before mating) and $Y = 0.03X - 1.92$, $r^2 = 0.202$, $P < 0.001$, $n = 335$ (after mating). The before-mating slope is larger than the after-mating slope ($t = 3.12$, $df = 669$, $P < 0.002$).

larger ejaculates to their second mate than did medium-sized males ($F_{1,9} = 16.63$, $P = 0.003$) (Fig. 8). Overall, a significant decrease in the combined weights of both female spermathecae occurred across all three matings ($F_{1,15} = 5.88$, $P = 0.028$). A significant decrease occurred

in the weight of large male ejaculates across all three matings ($F_{2,8} = 5.28$, $P = 0.034$), but not in the ejaculate weights of small ($F_{2,12} = 0.996$, $P = 0.398$) or medium ($F_{2,10} = 1.691$, $P = 0.233$) males. Ejaculate replenishment after the first mating (ejaculate weight in mating 2 minus

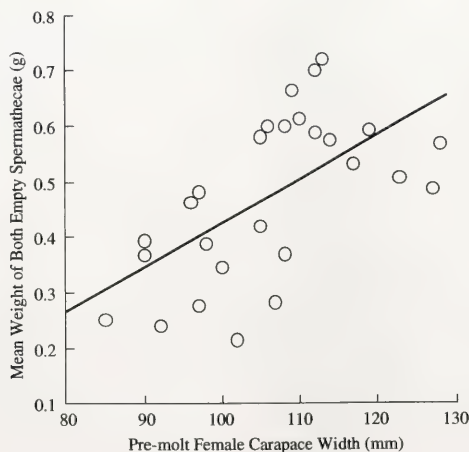


Figure 6. Relationship between unmated, pre-molt female carapace width and the mean weight of both empty spermathecae within the female. Females were isolated from males during the pubertal molt to ensure that their spermathecae were empty of male-derived material. $Y = 0.009X - 0.46$, $r^2 = 0.363$, $P = 0.001$, $n = 28$.

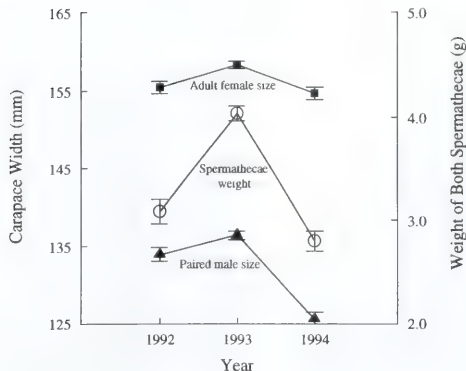


Figure 7. Sizes of adult females (■) and paired males (▲) and weight of female spermathecae (○) between 1992 and 1994. Results of Tukey comparison tests for differences between years are as follows: for females—1993 > 1992 ($P = 0.002$); 1993 > 1994 ($P < 0.001$), for males—1993 > 1992 ($P = 0.007$), 1993 > 1994 ($P < 0.001$) and 1992 > 1994 ($P < 0.001$); and for spermathecal contents—1993 > 1992 ($P < 0.001$), 1993 > 1994 ($P < 0.001$) and 1992 > 1994 ($P = 0.005$). Vertical bars are ± 1 standard error.

ejaculate weight in mating 1) increased with the interval between the first and second matings (Fig. 9) but not with that between the second and third matings ($n = 19$, $P = 0.588$). The interval between the first and second matings did not differ significantly from the interval between the second and third matings ($t = 1.99$, $df = 36$, $P = 0.056$).

Copulation duration

There was a significant effect of copulation duration ($F_{4,35} = 18.64$, $P < 0.001$) but not male size ($F_{2,35} = 0.544$, $P = 0.585$) on the amount of ejaculate passed to females. The interaction between male size and copulation duration was not significant ($F_{8,27} = 0.403$, $P = 0.909$). Males that copulated without interruption (8–12 h) passed larger ejaculates than males that were interrupted after 1, 2, or 4 h; males that were interrupted after 8 h passed larger ejaculates than males interrupted after 1 or 2 h (Fig. 10). No significant difference was found between the size of ejaculates passed by males that were interrupted after 8 h and that of uninterrupted males ($df = 37$, Tukey HSD = 0.319).

Discussion

In the field, paired males were larger than unpaired males in each year except 1994, and there was consistent, positive assortative mating between males and pre-molt females. The discrepancy in the results for 1994 may be the result of cold weather in that year, which retarded the growth of crabs and shortened the mating season. Consequently, the mean size of paired males (see Fig. 7),

the size range (54 mm CW) and maximum size (164 mm CW) of sexually mature males was low compared with other years: 1991, 74 mm CW and 179 mm CW; 1992, 89 mm CW and 184 mm CW; 1993, 101 mm CW and 204 mm CW. In blue crab and other crab, males use their chelipeds extensively during inter- and intrasexual interactions that lead to mating success, and the loss of one cheliped is a handicap in competition for females (Smith, 1992; Abello *et al.*, 1994; Paul and Paul, 1996). My results indicate that the chelipeds are proportionately longer in large male blue crab than in smaller ones; this length difference may, as seen in other species (Berrill and Arsenault, 1984; Carvacho, 1989; Homola *et al.*, 1991; Lee and Seed, 1992), provide a reach advantage during pair formation, and aggressive interactions for females, or both. Large males may also have advantages in maintaining their post-copulatory embrace, as indicated by the positive assortative mating with post-molt females in 1992 and 1993, because post-molt females were often larger than their male partner. In other crustaceans, small males often fail to remain paired with females that are larger than themselves (Adams, 1982; Verspoor, 1982; Adams and Greenwood, 1983; Forbes *et al.*, 1992). If female blue crab choose among potential mates, they may, as in other species, prefer large males for more protection from injury during takeover attempts (Smith, 1992) or from predation mortality during the female's soft, post-molt phase (Jivoff, 1997).

In each year, the relationship between male and female size in mating pairs was highly variable, suggesting that factors in addition to body size influence which individu-

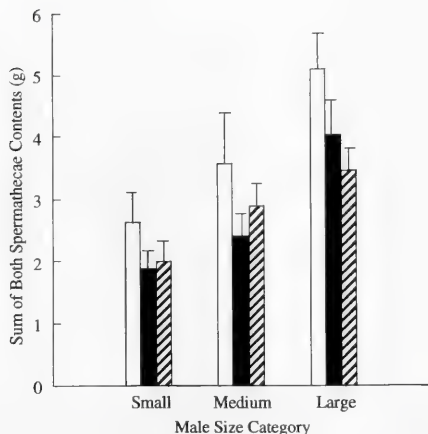


Figure 8. Sum of contents of both spermathecae from the first (□), second (■), and third (▨) females mated in succession by males in three size categories. The following are the results of paired contrasts between male size categories: first mating—large vs. small ($P = 0.009$), large vs. medium ($P = 0.182$), medium vs. small ($P = 0.338$); second mating—large vs. small ($P = 0.0002$), large vs. medium ($P = 0.003$), medium vs. small ($P = 0.272$); and third mating—large vs. small ($P = 0.036$), large vs. medium ($P = 0.359$), and medium vs. small ($P = 0.084$). Vertical bars are 1 standard error.

als are paired. In blue crab, a female's fecundity in a single brood increases with size, but the relationship is highly variable (Prager *et al.*, 1990), especially in comparison with other crab species (Hines, 1982). The number of broods female blue crab produce varies seasonally and

annually across the geographic range of the species (Chesapeake Bay: Provenzano *et al.*, 1983; Johnson and Hester, 1989; Jones *et al.*, 1990; von Montfrans *et al.*, 1990, South Carolina: Boylan and Wenner, 1993, Florida: Steele and Bert, 1994). Variation in fecundity per brood and

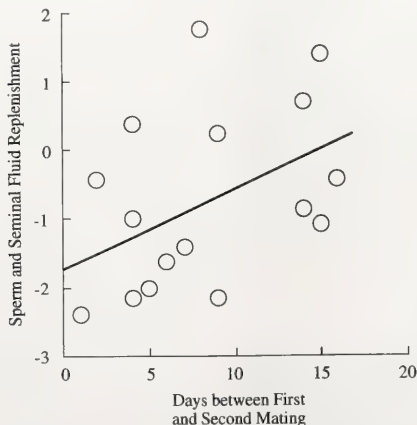


Figure 9. Relationship between ejaculate replenishment (total ejaculate passed in mating 2 - total ejaculate passed in mating 1) and the number of days between the matings. The regression equation is as follows: $Y = 0.14X - 2.01$, $r^2 = 0.243$, $n = 16$, $P = 0.038$.

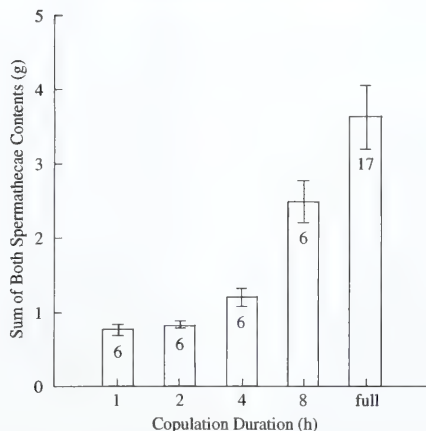


Figure 10. Effect of copulation duration on the amount of seminal fluid and spermatophores passed to both spermathecae. Results of a sample of uninterrupted matings (labeled "full") are also included. Numbers inside the bars are sample sizes. The differences are as follows: 4 h > 1 h ($P = 0.04$), 8 h > 1 h ($P < 0.001$), full > 1 h ($P < 0.001$) and 8 h > 2 h ($P = 0.001$), full > 2 h ($P < 0.001$), full > 4 h ($P = 0.014$). Vertical bars are ± 1 standard error.

total brood production suggests that the fecundity benefits conferred by large females are uncertain. Female molt stage also influences which individuals are paired by affecting both the ease with which males can establish mate guarding and its duration (Jivoff and Hines, in press). Large females take longer to progress through the final molt cycle than smaller females (Smith, 1997), thus males specializing in large females may mate guard for the longest durations. A simulation model (Jivoff, 1995) shows that male blue crab can enhance their seasonal reproductive success by pairing for shorter durations, implying that late pre-molt females of any size may be preferred over large females in earlier molt stages. Thus, factors that influence both a male's mating frequency and his fertilization rate per female may regulate male mating success in blue crab.

The experimental results indicate that large males pass larger ejaculates to each of their mates than do small males. In the field, females held more material in their spermathecae when large males were more often in mating pairs; this observation also suggests that large males provide females with larger ejaculates. In a variety of other species, large ejaculates ensure that more eggs are fertilized (Gwynne, 1984; Woodhead, 1985; Simmons, 1988; Wiklund *et al.*, 1993) and enhance a male's ability to compete for unfertilized eggs if the female remates (Gromko *et al.*, 1984; Gage, 1991; Lewis and Austad, 1994; Eady, 1995). My field results suggest that large males have access to more unfertilized eggs by means of

size-assortative mating, therefore they may enhance their fertilization rate by passing large ejaculates. Previously, I have shown that large ejaculates may provide males with disproportionate access to a female's unfertilized eggs if she remates (Jivoff, 1997). The experimental results indicate that large males pass large ejaculates because they have greater stores of spermatophores and seminal fluid, because they can copulate for at least 8 h, or both. In other species, large males may copulate for longer durations (Ward and Simmons, 1991), because they can resist disruptions and displacement during copulation (Berrill and Arsenault, 1982; Abele *et al.*, 1986; Reid *et al.*, 1994; Hazlett, 1996). I have no size-related measures of copulation duration; however, large male blue crab prevent aggressive displacement by other males during mate guarding (Smith, 1992; Jivoff, 1995) and therefore may copulate longer than small males.

Ejaculates may be costly for males to produce, and the number of sperm per ejaculate may influence a male's paternity, so males should allocate their ejaculates among females, and adjust the size of ejaculates to ensure high levels of paternity (Dewsbury, 1982; Parker, 1990). In a single mating, males passed an average of 47% of their stored seminal products, but when males, especially large ones, mated in rapid succession there was a decrease in the size of their ejaculates. This decrease suggests that one cost of passing consistently large ejaculates is an increase in the time needed to replenish seminal products. One experiment provided an indirect measure of ejaculate

replenishment on the basis of the size of successive ejaculates, but it may also be important to control the time between successive matings. In blue crab and other portunids, replenishment of a male's sperm and seminal fluid may be incomplete if the interval between successive copulations is less than 15 days (see Fig. 9) (Ryan, 1967). In blue crab (Jivoff, 1995) and other crustaceans (Manning, 1980), males respond to increased numbers of competitors with longer periods of pre-copulatory mate guarding. This response ensures a male's access to a female, and by increasing the time between matings, may also provide time for replenishment or build-up of ejaculate supplies. Large ejaculate stores may be an advantage in the presence of rivals because the risk of sperm competition increases, and male blue crab respond to that risk by passing larger ejaculates (Jivoff, 1997). A larger ejaculate may enhance a male's fertilization rate, even if the female remates, because each inseminator's ejaculate appears to have access to the unfertilized eggs (Jivoff, 1997).

Male blue crab compete for access to mates and perhaps, through sperm competition, for the unfertilized eggs of the female. My results suggest that large body size gives a male an advantage in both forms of competition; however, the variability in both the degree of assortative mating and the investment in ejaculate contents by males of different size suggests that male size is but one factor influencing male mating success. Male blue crab appear to have responded to sexual competition with both pre- and post-copulatory mate guarding, therefore the factors that influence the duration of both types of mate guarding may interact to influence male mating success. The results suggest that the time spent in pre-copulatory mate guarding may influence a male's ability to replenish his supply of sperm and seminal fluid, which may, in turn, affect his ability to compete during sperm competition.

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Symbiosis of the Hydrothermal Vent Gastropod *Ifremeria nautilei* (Provannidae) With Endobacteria—Structural Analyses and Ecological Considerations

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Abstract. The gastropod *Ifremeria nautilei* lives in high abundance around deep-sea hydrothermal vents of the Western Pacific. The filaments of its ctenidium are very long and have a rigid axis with a hemocoelic vessel and a strongly ciliated epithelium. The flattened part of each filament largely consists of bacteriocytes that are distally filled with numerous gram-negative bacteria. The bacteria lie one by one in vacuoles that seem to be part of an interconnected tubular system. Some of the apical vacuoles regularly showed what could be openings to the ambient seawater. This special topological arrangement of the bacteria suggests that in a morphological series mirroring the supposed evolutionary pathway from extra- to intracellular symbioses, *I. nautilei* might correspond to an intermediate stage.

The high sulfur content and the low stable carbon isotope values measured in this study, combined with corresponding data from the literature, indicate that *I. nautilei* is the host partner in a thiotrophic chemoautotrophic bacterial symbiosis. The importance of this symbiosis for the nutrition of the gastropod is underlined by the reduced size of the host's stomach. Unlike specimens of *I. nautilei* from the Manus Basin (Galchenko *et al.*, 1992), the inspected specimens from the North Fiji Basin did not contain any methanotrophic bacteria in addition to the thiotrophic type. From the disparity in results, it may be con-

cluded that this host species can develop different patterns of symbiosis either as an adaptation to local variances of hydrothermal vent fluid chemistry or as a consequence of genetic differentiation in the host.

Introduction

Hydrothermal vents, which have been known since the late 1970s, have become the subject of intensive study. Biological investigations have concentrated on vents in the East Pacific and on the Mid Atlantic Ridge, where vestimentiferans, bivalve molluscs, and shrimps dominate the ecosystems (Van Dover, 1990; Tunnicliffe, 1991). It has been shown that symbioses between most of these animals and chemoautotrophic bacteria are the main source of nutrition (Fisher, 1990; Childress and Fisher, 1992). The animals supply the bacteria with reduced compounds from the vents and with oxygen from the ambient seawater. The bacteria gain energy by oxidizing sulfur species—and sometimes methane as well (Childress *et al.*, 1986; Fisher *et al.* 1987, 1993; Cavanaugh *et al.*, 1987; Distel *et al.*, 1995)—for the fixation of organic carbon which, in turn, is utilized by their hosts.

Only in hydrothermal vent ecosystems of the Western Pacific are gastropod molluscs abundant, even attaining a dominant ecological role (Van Dover, 1990; Tunnicliffe, 1991). To date, vent areas have been investigated in the Lau Basin (Desbruyères *et al.*, 1994), the Manus Basin (Both *et al.*, 1986; Tufar, 1990; Galkin, 1992), the Mariana Trough (Hessler *et al.*, 1988; Hessler and Lonsdale, 1991), and the North Fiji Basin (Jollivet *et al.*, 1989;

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Desbruyères *et al.*, 1994). In almost all these areas, one or both of the two prosobranch gastropods *Ifremeria nautilei* and *Alviniconcha hessleri* (Family Provannidae) occur in abundance together with several mytilid bivalve species of the genus *Bathymodiolus* (Warén and Bouchet, 1993; Desbruyères *et al.*, 1994; Cosel and Métivier, 1994). The gastropods settle in the vicinity of hydrothermal emissions where they are exposed to temperatures of about 3°–20°C.

Alviniconcha hessleri was the first hydrothermal vent gastropod reported to contain endosymbiotic bacteria within its specialized gills (Ohta *et al.*, 1988; Stein *et al.*, 1988; Endow and Ohta, 1989a, b). The high content of elemental sulfur, the autotrophic fixation of CO₂, and the high activity of enzymes that catalyze sulfide oxidation indicated that the bacteria were chemoautotrophic sulfur-oxidizing endosymbionts (Stein *et al.*, 1988). But Ohta *et al.* (1988) and Endow and Ohta (1989a, b) found in one of the three specimens of *Alviniconcha hessleri* inspected a second morphological type of bacterial symbionts closely resembling methanotrophic bacteria.

In micrographs of *A. hessleri* (Stein *et al.*, 1988), bacteria appear to be intracellular and enclosed singly in host vacuoles, although some vacuoles with several bacteria can be seen. But Endow and Ohta (1989a, b) noted that the apical vacuoles of several bacteriocytes contained narrow ducts that seemed to directly connect the bacteria to the exterior. On the other hand, they demonstrated by infiltration of ruthenium red that in the basal parts of the bacteriocytes the symbionts were truly enclosed in their vacuoles (Endow and Ohta, 1989b).

Ifremeria nautilei, the other hydrothermal gastropod species from the Lau and North Fiji Basins (Bouchet and Warén, 1991; Beck, 1991; Warén and Bouchet, 1993), deviates in several anatomical details from the related provannid *A. hessleri*. The hypertrophic gills, the well-developed circulatory system, and the reduced stomach lead to the assumption that symbiotic bacteria might play a more significant role in these animals. Galchenko *et al.* (1992), examining specimens from the Manus Basin, provided the first physiological evidence for a bacterial symbiosis. CO₂-fixation was stimulated by reduced sulfur compounds, and radiolabeled CH₄ was metabolized and detected in the tissues. These results suggested the presence of thioautotrophic and methanotrophic bacteria in the gills, providing the gastropod with reduced carbon. The ultrastructural basis given by the authors for these results was very preliminary, and the number of specimens or tentacular filaments inspected was not indicated. The micrographs supported the physiological data, reinforcing the conclusion that two different physiological pathways were being used by two morphologically distinct types of bacteria. Specimens of *I. nautilei* from the

Lau and North Fiji Basin have only been studied physiologically: Desbruyères *et al.* (1994) confirmed the presence of thiotrophic symbionts by the detection of ribulose-1, 5-bisphosphate carboxylase activity.

The present study on *Ifremeria nautilei* from the North Fiji Basin concentrates on an (ultra)structural survey of the bacterial symbiosis to scrutinize important details previously not sufficiently documented (Galchenko *et al.*, 1992). These structural results are supplemented by measurements of elemental sulfur, total sulfur, and stable carbon isotope composition. They give further insight in the trophic interactions between the endosymbiotic prokaryotes and their gastropod host.

Materials and Methods

During cruise 99 of the RV *Sonne* in January 1995 (Auzende *et al.*, 1995; Halbach *et al.*, 1995), many specimens of *Ifremeria nautilei* (Bouchet and Warén, 1991) were collected in the North Fiji Back Arc Basin at the LHOS site (Ishibashi *et al.*, 1994) at 16°59.65 S–173°54.73 E. The sample was retrieved from a depth of 2000 m (station GTV 115) by a TV-controlled grab. The grab also contained the symbiont-harboring bivalve *Bathymodiolus brevior* and other hydrothermal animals. The specimens of *I. nautilei* examined had a shell height of about 5 cm. Five gill filaments from each of two specimens were dissected and fixed in a mixture of 4% paraformaldehyde, 3% glutaraldehyde, and 15% sucrose in 0.1 M cacodylate buffer (pH 7.4). After being rinsed in the same buffer, the tissue was postfixed in OsO₄ (1%). Samples were dehydrated in acetone, embedded in Spurr's resin, and sectioned for light and electron microscopy. For light microscopy, semithin sections were stained with toluidine blue; for electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate. Sections of filament from the two specimens were examined using a Zeiss 902A electron microscope. One filament was sequentially cut and, in intervals of 100 µm, ultrathin sections were examined. For scanning electron microscopy, several gill filaments of two formaldehyde-fixed (4%) specimens were dehydrated in acetone, critical-point-dried, and gold-sputtered. A Cambridge Camscan DV 4 scanning electron microscope was used for the observations. For paraffin sections, a complete specimen was fixed in 4% formaldehyde. After dissection and standard embedding, sections were cut and stained with Azan (Romeis, 1989).

Measurements of total sulfur (S^{total}) concentration were based on samples from three individuals (10 to 40 mg dry weight). The lyophilized gill and muscle tissues of the foot (excluding the warts, Warén and Bouchet, 1993) were digested separately for 3 h under pressure at 130°C

in 4 ml HNO_3 (65%, suprapur, Würfels and Jackerth, 1985). The resulting solution was concentrated by evaporation to 0.5 ml, then brought to 14 ml with distilled water. In this solution the sulfur concentration was measured with an inductivity-coupled-plasma atomic emission spectrometer (ICP-AES, *Perkin Elmer Plasma II*). Oyster tissue SRM 1566, commonly used in mollusc studies, and a diluted standard solution were used for calibration.

For measurements of elemental sulfur (S^0), frozen gill and foot muscle tissues of three individuals were lyophilized and analyzed by HPLC (pump: Jasco 880 PU, manual injector: Rheodyne 7105, fitted with a 20 μl loop; UV/VIS detector: Jasco 975-UV, set at 254 nm; column: Hamilton PRP-1 reversed phase (15 cm $\times$ 4.1 mm I.D.). The absorbance was continuously collected by a computer using the Labtech notebook software. Chromatograms were analyzed using the Labtech chrom software. Treatment of the column prior to analysis, extraction of samples in chloroform, and HPLC-protocol were according to Lauren and Watkinson (1985).

The $\delta^{13}\text{C}$ was measured with a Finnigan Delta E mass

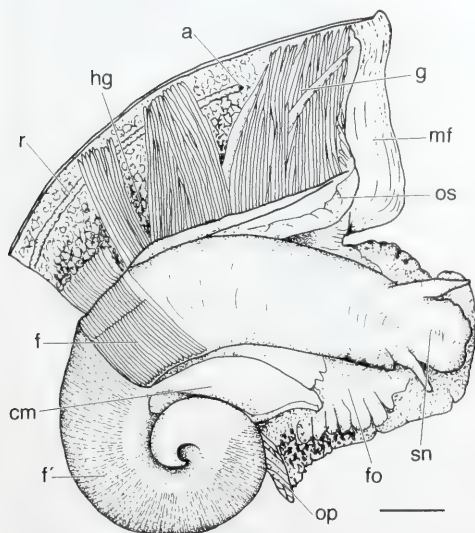


Figure 1. *Ifremeria nautiliei*. Anatomy of the mantle cavity and the ctenidium to show the dimension and location of the ctenidium. The shell has been removed, the mantle cavity partly opened, and in "f" the basal attached part of the filaments dissected from mantle. a, anus; cm, columellar muscle; f, gill filaments; f', gill filaments inside the mantle cavity; fo, foot; g, gill; hg, hypobranchial gland; mf, mantle fringe; op, operculum; os, osphradium; r, rectum; sn, snout. Scale 10 mm (after Beck, 1991, modified).

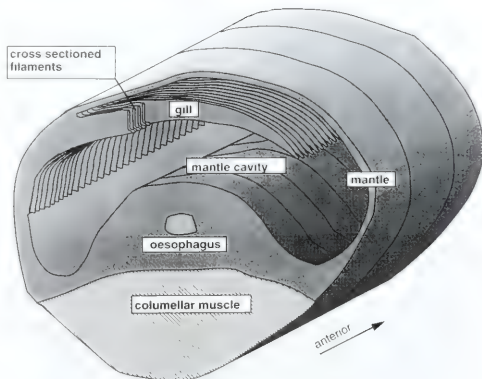


Figure 2. Schematic drawing of interior anatomy showing position and arrangement of the ctenidium in the mantle cavity.

spectrometer from gill tissue of three specimens. After decalcification with 0.1 N HCl and drying in an oven at 60°C, samples were combusted, at 1050°C in a stream of oxygen, to CO_2 by using a commercial Heraeus CHN analyzer attached to the mass spectrometer. Stable isotope determinations were expressed as the permille difference from the international Pee Dee Belemnite standard, where $\delta^{13}\text{C} = [\text{Ratio}_{\text{sample}} / \text{Ratio}_{\text{standard}} - 1] \times 10^3$ (‰).

Results

Anatomy of the gill

Ifremeria nautiliei has a single ctenidium at the left side of its body, where it occupies about one whorl of the snail's shell (Fig. 1). The ctenidium consists of 1000 to 2000 serially arranged slender filaments with a length of 9 to 17 mm and a maximal width of about 0.1 to 0.2 mm. Towards their proximal and distal ends, these filaments decrease in length and width. The basal third of each filament is attached to the roof of the mantle cavity and the remaining two thirds extend free into the mantle cavity (Fig. 2). The leaflike main part of the filament is thin, its free lower edge, orientated towards the mantle cavity, is reinforced by a rigid axis (Fig. 3A). In some preparations the axis was found bent towards the anterior end of the animal, resulting in an L-shaped filament (Fig. 4); this curvature may be dependent on muscle contraction or fixation. The following description is based on cross sections through the nonattached part of a ctenidial filament.

Each filament consists of two epithelial cell layers separated by a bilayered middle lamella of noncellular material (Figs. 3, 4). The internal space between these lamellar

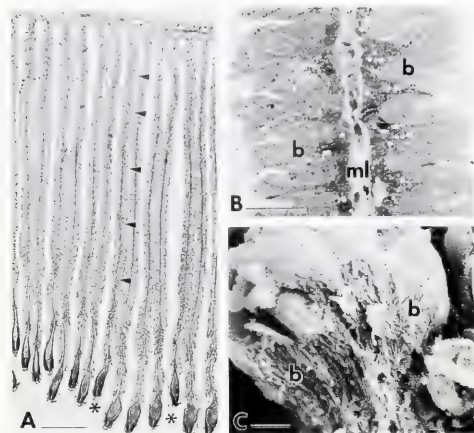


Figure 3. (A) Cross section through several attached ctenidial filaments. Nearly the entire filament consists of bacteriocytes (arrowheads). The ventral edge (*) contains a wide hemocoelic vessel; the dorsal edge is attached to the mantle (azan-stained paraffin section, light micrograph). Scale 1 mm. (B) Details of filament, median cross section, showing the two layers of bacteriocytes (b) separated by the middle lamella (ml). Scale 100 μ m. (C) Scanning electron micrograph of bacteriocytes with cell wall peeled open showing the dense packing of bacteria (b). Scale 5 μ m.

layers (width varying between 2 and 15 μ m) harbors muscles, nerves, connective tissue, hemocoel with hemocytes, and additional extracellular material. At the distal, ventral edge of the filament the two layers of extracellular material become very thick (50 μ m) and join, enclosing a wide hemocoelic vessel that extends all along the lower edge of the ctenidial length. In fixed material the hemocoelic space appears more or less empty, containing only some granular material, possibly remnants of the blood fluid (Fig. 5A). At the boundary walls, very flat cells are abundant (Fig. 5C).

Each cross section through a filament (dorsal to ventral, see Fig. 2) can be subdivided into six zones, five of which represent the symbiont-free parts, the remaining large one the symbiont-containing median part of the ctenidial filament (for numbering see Fig. 4). Zones 1–4 form the lower (ventral) axis of the filament, zone 6 the upper (dorsal) edge.

Symbiont-free parts. The five zones that lack symbionts are described below.

Zone 1—the ventral edge of the filament (Fig. 5A). It consists of a ciliated epithelium with cells having a height of 10 to 20 μ m and containing large nuclei (5 μ m long). Externally, they are covered by 1.25 μ m long microvilli and by several 5 μ m long cilia. Glandular cells are abundant

and contain electron-lucent vacuoles that harbor several homogeneous granules. Underneath the epithelium several small nerves traverse the filament (arrows in Fig. 5A).

Zone 2—the zone enclosing the vessel (Fig. 5B). Here, flat (about 4 μ m high) nonciliated epithelial cells form a very thin cellular cover on the extracellular material. The cells contain only little electron-dark cytoplasm; at their external side they are studded with microvilli 0.5 to 0.7 μ m in length. The mitochondria are electron dense, their cristae rarely visible.

Zone 3—the zone of strong ciliation (Fig. 5C). It consists of two layers of large, strongly ciliated cells (15 μ m high), based on thin (1 μ m) noncellular lamellae that en-



Figure 4. Median cross section of a ctenidial filament. Drawing compiled from light and electron microscopic observations. Bacteriocytes are the dominant cell type. Numbers indicate "zones" of different epithelia as described in the text.

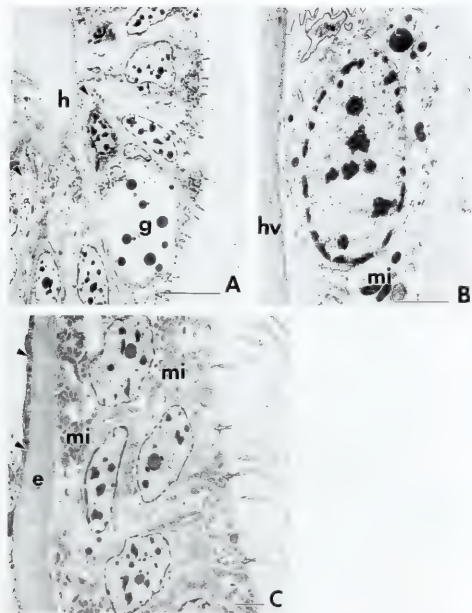


Figure 5. Cross section through ctenidial filament with various types of epithelia; see Figure 4 for zone designation. (A) Zone 1. Ciliated epithelium from the ventral edge of a filament with a gland cell (g), h = hemocoel, arrowheads = nerves. Scale $5\ \mu\text{m}$. (B) Zone 2. Flat, nonciliated epithelium covers the hemocoelic vessel (hv). Mitochondria (mi) have, as in all other cells of the gill, a very electron-dark matrix. Scale $2\ \mu\text{m}$. (C) Zone 3. Strongly ciliated epithelium with many mitochondria (mi) and an underlying thick layer of extracellular material (e), arrowheads = thin layer of flat cells. Scale $1\ \mu\text{m}$.

close a separating space about $2\ \mu\text{m}$ in width. This lacunar space is nearly completely filled with cells that probably represent hemocytes (not shown in micrograph). In each ciliated cell three areas can be recognized: The apical area, $3.5\ \mu\text{m}$ high, is characterized by abundant cilia up to $20\ \mu\text{m}$ long. Here, in addition to mitochondria, the cytoplasm contains rough endoplasmic reticulum and spherical, electron-dense vacuoles $0.5\ \mu\text{m}$ in width. The middle area of the cells is dominated by globular, large nuclei of about $3.5\ \mu\text{m}$ diameter. The basal cell area is completely filled with tightly packed oval mitochondria. These are about $0.5\ \mu\text{m}$ in length and contain many parallel, well-developed cristae.

Zone 4—the hinge zone (Fig. 6A). Located between the ciliated cells (zone 3) and the bacteriocytes (zone 5), this region is characterized by large ($15\ \mu\text{m}$ high), nonciliated cells. In comparison to the surrounding tissue,

here both the cell membranes and the thin ($0.5\ \mu\text{m}$) extra-cellular lamellae are strongly folded. This might indicate the function of this zone as a hinge between the axis and the rest of the filament. The two central lamellae are separated by an interlamellar space that is about 15 to $20\ \mu\text{m}$ in width and traversed by two groups of muscles, two large nerves, and a hemocoelic lacuna. Minor nerves are located at the base of the epithelial cells.

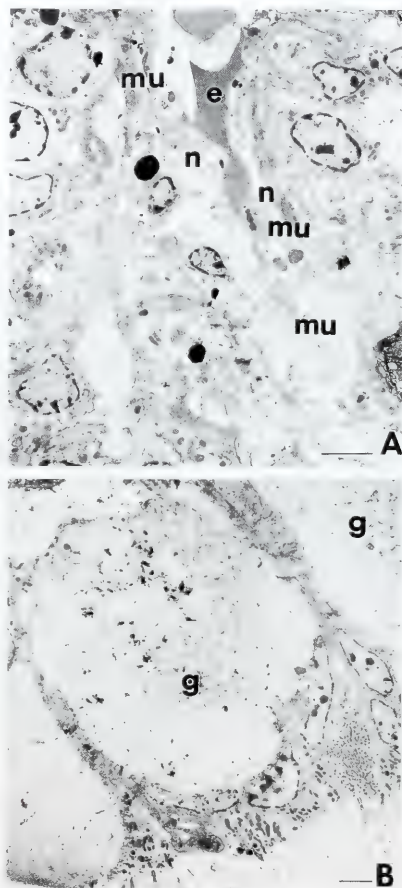


Figure 6. Cross section through ctenidial filament with various types of epithelia, continued; see Figure 4 for zone designation. (A) Zone 4. A "hinge" epithelium with a well-developed middle lamella in the center where nerves (n), muscles (mu), and extracellular material (e) are abundant. Scale $2\ \mu\text{m}$. (B) Zone 6. Ciliated epithelium with prominent gland cells (g). Scale $2\ \mu\text{m}$.

Zone 6—the upper filament edge (Fig. 6B). In the nonattached part of the tentacular filament this zone forms the dorsal edge, characterized by an epithelium consisting of glandular and ciliated cells. Three morphotypes of glandular cells with apparently different secretions can be distinguished. Interspersed between these glandular cells are intercalary, multiciliated cells with cilia 5 μm long.

Bacteriocytes and bacterial symbionts in tentacular filament. The symbiont-containing median part of the tentacular filament is Zone 5.

Zone 5—the symbiont zone (Figs. 3, 4, 7A, 9). Representing nearly the entire flattened part of the filament, this dominant region is characterized by numerous symbiont-containing epithelium cells (bacteriocytes). About 250 of these cylindrical to pear-shaped bacteriocytes (about 30

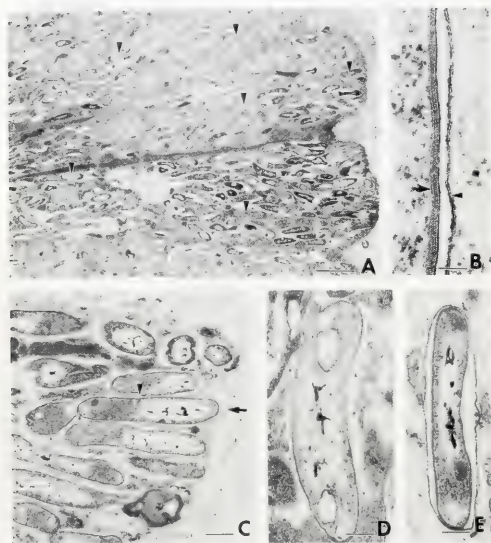


Figure 7. (A) Longitudinal section of the distal part of a bacteriocyte demonstrating the dense packing of bacteria (arrowheads). Only one morphological type of bacterium is observed. Scale 2 μm . (B) Cell envelope of the bacterium with a typical gram-negative pattern (arrow) and the adjacent unit membrane (arrowhead) of the host cell. Scale 50 nm. (C) Typical parallel arrangement of bacteria in the apical region of a bacteriocyte. Note anastomoses in vacuoles containing bacteria (arrowhead) and the opening of one vacuole to the outside (arrow). Scale 0.5 μm . (D) Bacterium containing membrane-bound electron-lucent vacuoles. Scale 0.25 μm . (E) Rod-shaped bacterium with peripherally arranged granular plasma and centrally condensed DNA, but without vacuoles. Scale 0.25 μm . Micrograph D is from a specimen with high S° content in the gill; E from a specimen with low S° in its gill.

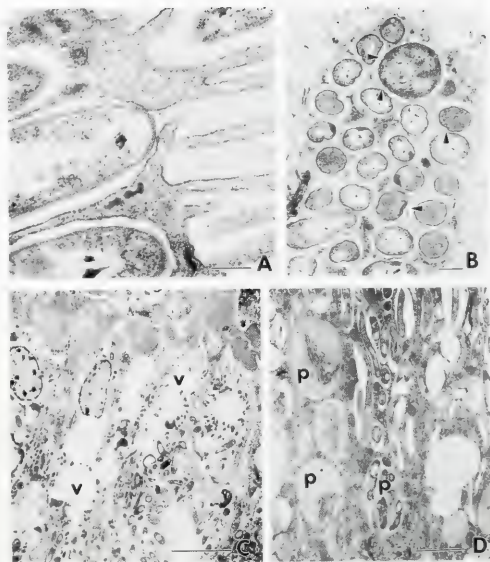


Figure 8. (A) Opening of a bacterium-containing vacuole to the outside of the cell. Scale 0.2 μm . (B) Apical tip of bacteriocytes in cross section showing anastomoses (arrowheads) between the bacterium-containing vacuoles. Scale 0.5 μm . (C) Basal part of bacteriocytes. Abundant non-membrane-bound electron-lucent vacuoles (v) whose content might have been partly extracted during preparation. Scale 5 μm . (D) Basal part of bacteriocytes. Structures resembling phagolysosomes (p) in different stages. Scale 2 μm .

to 50 μm long, maximum diameter about 8 μm) are visible on each cross section through the filament.

The cytological structure of the bacteriocytes is fairly uniform. Their surface is studded with 0.7 μm -long microvilli. The outer parts of these cells are densely filled with inclusions that resemble bacteria (Figs. 3C, 7, 8). Most are about 2.5 μm (length) by 0.5 μm (width), but some may reach a size of 4 μm by 1 μm . Their cell envelope is of the gram-negative type with clearly visible inner and outer membranes (Fig. 7B). A peripherally located dense cytoplasm and a central network of condensed DNA betray the prokaryotic nature of these cells (Figs. 7C, D, E). In one of the two specimens inspected the bacteria contained membrane-bound electron-lucent vesicles (Fig. 7D). Typical bacterial division stages were not found. Since transitional stages between all bacterial sizes were found, there are no reasons to assume the existence of different morphological types of bacteria in the examined sections. Membrane stacks, characteristic for the methanotrophic bacteria, could never be discerned.

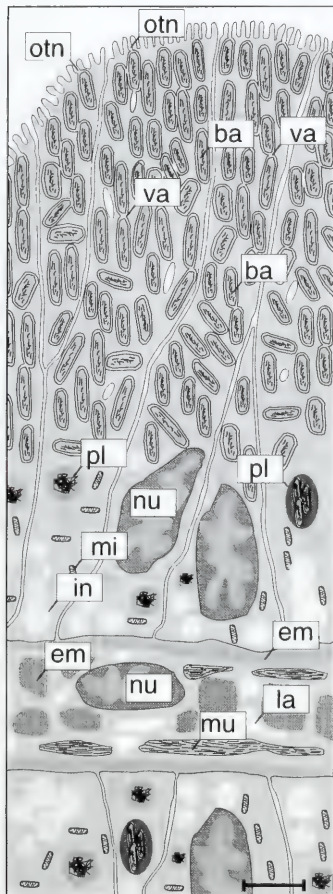


Figure 9. Schematic representation of bacteriocytes and underlying middle lamella. ba, bacteria; em, extracellular material; in, electron-lucent inclusion; la, hemocoelic lacuna; mi, mitochondrion; mu, muscle; nu, nucleus; otn, opening of apical bacterial vacuoles; pl, phagolysosome-like structure; va, bacteria-containing vacuoles. Scale 5 μ m.

Each bacterium was tightly embedded in a vacuole, but the vacuoles often appeared interconnected. Therefore there seems to exist a complex "network" of anastomosing vacuoles harboring the bacteria. (Figs. 7C, 8B). Moreover, in all the examined sections, some peripheral vacuoles were observed to have an opening towards the outside environment (Figs. 7C, 8A).

Whereas the distal part of the bacteriocytes contained

only bacteria and a few mitochondria, the basal part was dominated by the nucleus and several large, non-membrane-bound droplets of a homogeneous, electron-lucent material (Fig. 8C). This internal cell area also harbored many 1 μ m-long electron-dark mitochondria that were rich in cristae. Inclusions resembling phagolysosomes (Fiala-Médioni *et al.*, 1994), which would represent stages of bacteria in digestion, could often be observed in the basal part of the bacteriocytes (Fig. 8D).

Interspersed between the bacteriocytes, but in much lower abundance, were secretory cells and a few intercalary cells. The two lamellae between the epithelial layers of bacteriocytes enclosed a space that was filled by hemocoel, putative hemocytic cells, thick cords of collagen, and connective tissue (Figs. 3B, 6A).

Sulfur and ^{13}C measurements

In the gill and foot tissue of *Ifremeria nautilei*, sulfur was detected (Table I) in concentrations comparable to those in other symbiont-containing molluscs, but somewhat lower than those reported for Vestimentifera (see compilation in Stein *et al.*, 1988). Concentration of elemental sulfur (S^0) differed by a factor of 8 among the individuals examined. The average concentrations of S^0 and S^{total} were significantly higher in the gill than in the foot (Table I).

The stable carbon isotopic values measured from three specimens of *I. nautilei* equaled -31.4‰ , -31.4‰ , and -33.7‰ . Thus, they were much more depleted than those in marine animals that are linked to a photosynthetically based food chain (Fry and Sherr, 1984).

Table I

Percentages ($\pm$ SD) of elemental sulfur (S^0) and total sulfur (S^{total}) (based on mg dry weight) in gill and foot of three individuals (115-1, 115-2, 115-4) of *Ifremeria nautilei*

Sample	Organ	S^0	S^{total}
115-1	gill	0.54 ± 0.02	2.23
	foot	0.25 ± 0.21	1.43
115-2	gill	3.98 ± 0.93	2.49
	foot	0.13 ± 0.13	1.14
115-4	gill	1.88 ± 0.14	2.84
	foot	0.03 ± 0.01	1.05

Values for S^0 and S^{total} are significantly higher in the gill than in the foot (Mann-Whitney *U*-test). In gill sample 115-2, the higher value of S^0 than S^{total} may account for the patchy localization of elemental sulfur in the tissue. Additionally, the resulting variance of data may be altered by the different sample sizes required for the two methods of sulfur identification.

Discussion

Comparison with other provannid gastropods

The first hydrothermal vent gastropod shown to contain thiotrophic bacteria in its gills was *Alvinicoconcha hessleri* (Stein *et al.*, 1988). This provannid species is often found around the same hydrothermal outlets as *Ifremeria nautilei*, to which it is closely related (Both *et al.*, 1986; Jollivet *et al.*, 1989; Tufar, 1990; Galchenko *et al.*, 1992; Desbruyères *et al.*, 1994). Despite some differences in gross morphology—*e.g.*, in shell structure, nervous system, and pallial margin, as well as in the presence of oesophageal pouches and wartlike tubercles on the foot of *L. nautilei* (Warén and Bouchet, 1993)—the two species apparently have a very similar type of bacterial symbiosis. This conclusion is based on ultrastructural data from the preliminary work by Galchenko *et al.* (1992) and from the present study.

An array of structural features of *I. nautilei* indicates that the bacterial symbiosis is of high functional importance for the host.

- The most obvious trait is the extremely high number and unusual length of ctenidial filaments. *I. nautilei* has 15 to 20 times more ctenidial filaments than the 30 to 89 found in its aposymbiotic relative *Provanna* spp. (Warén and Ponder, 1991).

- The ctenidial filaments in monopectinate prosobranch gastropods are usually triangular, with their base fully attached to the mantle roof (Demian, 1965). A ciliary ridge extending from the efferent edge of the filaments creates water currents for irrigation (Lutfy and Demian, 1965). In *I. nautilei* this efferent edge is highly elongated and a rigid axis has developed that bears the extended bacteriocytic epithelia and encloses a wide hemocoelic vessel. This axis may enhance the stability of the flimsy leaflike filaments. It also may act as a flexible hinge that, in cooperation with longitudinal and transverse muscle fibers, would allow movement of the whole filament. The "blood" vessel is presumably responsible for nutrition and gas exchange.

- The bacteria occur in extremely high numbers in the bacteriocytes and occupy a large area of the filaments. The stomach of the host is reduced to only $1/10$ the size of the stomachs of related nonsymbiotic provannid gastropods (Bouchet and Warén, 1991). This points to the relevance of the symbiosis for the animals' nutrition. Furthermore, even though bacteriocytes represent the dominant structures in the gill tissues of other molluscs with a bacterial symbiosis, the bacterial zone in *I. nautilei* is nearly twice as voluminous as in these species (Le Pennec and Hily, 1984; Southward, 1986; Fiala-Médioni *et al.*, 1986; Fiala-Médioni and Métivier, 1986; Fiala-Médioni, 1988; Fiala-Médioni and Felbeck, 1990). Whether this differ-

ence mirrors a larger dependence on chemoautotrophic nutrition is an open question.

The black-tipped warts on the foot of *I. nautilei* have been suspected, without closer analysis, to be "involved" in the symbiosis (Warén and Bouchet, 1993). Our analyses of ultrathin sections through the warts (data not shown) did not demonstrate the presence of any bacteria, thus refuting such an assumption.

Position of the bacteria in a vacuolar network

In most molluscs with chemoautotrophic bacteria, the prokaryotes are intracellular although their detailed arrangement may vary. In *I. nautilei* the position of the symbiotic bacteria in vacuoles of ctenidial cells is essentially the same as that described by Endow and Ohta (1989a, b) for the related gastropod *A. hessleri*. These studies indicate that peripheral vacuoles have some contact to the ambient seawater via small ducts. This topological arrangement seems to be specific to these two related gastropod species and might indicate a common origin of the symbiosis. In *A. hessleri* it could be demonstrated by staining with ruthenium red that this connection via "open" vacuoles was restricted to the externally located bacteria (Endow and Ohta, 1989b). Regrettably, the fresh material needed for this experiment was not retained in *I. nautilei*.

The chemoautotrophic nature of the bacteria

In specimens of *I. nautilei* from the Lau and Fiji Basins (Desbruyères *et al.*, 1994) and from the Manus Basin (Galchenko *et al.*, 1992), ribulose-1, 5-bisphosphate carboxylase/oxygenase activity provided evidence for the chemoautotrophy of the symbiotic bacteria. The stable carbon isotope values measured for *I. nautilei* in this study are higher than those reported for specimens from the Manus Basin (Galchenko *et al.*, 1992). They stay in the range of values found in symbiont-containing bivalves known to depend fully on their symbionts for nutrition (Childress and Fisher, 1992). Supporting evidence for the thiotrophic nature of the symbionts is provided by the high concentration of sulfur in the gills; this is regarded as a reliable criterion for the presence of sulfur-oxidizing bacteria (Childress and Fisher, 1992). It is, of course, still possible that this sulfur is a product of purely chemical oxidation of sulfide. The concentration of elemental sulfur in *I. nautilei* (0.5%–3.9% dry weight) is comparable to that in the gastropod *A. hessleri* (Stein *et al.*, 1988) or in the bivalves *Lucinoma annulata* (0.2%–2.1% dw; Vetter, 1985) and *Calyptogena magnifica* (4.4% dw, Stein *et al.*, 1988). Similarly, the concentration of total sulfur (2.2%–2.8% of dry weight) in the gills of *I. nautilei* is in the same range as in the gills of *L. annulata* (2.5%–5.6%

dw; Vetter, 1985) and *C. magnifica* (3.8% dw; Roesijadi and Creelius, 1984) which have been proven to harbor thiotrophic bacteria. The foot, which has never been found to harbor bacteria in any mollusc with bacterial symbionts, contained about 1% dry weight total sulfur in *I. nautili* (see Table 1).

The regular occurrence of degradation stages of symbiotic bacteria could indicate a digestive utilization of the prokaryotes by their host. This "harvesting" via phagocytotic degradation has also been found in *Riftia pachyp-tila* (Bosch and Grassé, 1984), in symbiont-harboring oligochaeta (Giere and Langheld, 1987), and in bivalves (Fiala-Médioni and Felbeck, 1990).

Thiotrophic vs. methanotrophic bacteria

Methanotrophic bacteria have been reported in *I. nautili* from the Manus Basin (Galchenko *et al.*, 1992). The identification was based on the activity of the methanol-oxidizing enzyme methanol dehydrogenase, the incorporation of labeled carbon from methane, the very low $\delta^{13}\text{C}$ value of the gills (-38.3‰), and the presence of intracytoplasmic membranes in some of the bacteria. This is in contrast to the present study on specimens of the same species retrieved in the Fiji Basin, some 3000 km away. We could not discover any bacteria with the internal membranes typical of methanotrophic bacteria (Childress *et al.*, 1986; Fisher *et al.*, 1987; Cavanaugh *et al.*, 1987; Cavanaugh, 1992; Fisher *et al.*, 1993; Distel *et al.*, 1995). Although, at least in free-living methanotrophs, the occurrence of membranes is variable depending on culture conditions (Prior and Dalton, 1985; Collins *et al.*, 1991), the higher $\delta^{13}\text{C}$ values found in our specimens might also indicate an absence of methanotrophs. For a more conclusive interpretation, the $\delta^{13}\text{C}$ values of the methane sampled at the North Fiji hydrothermal vents are required.

Our samples, taken from only a few specimens and ctenidial filaments, might have missed material containing methanotrophic bacteria due to an inhomogeneous distribution in the large gills. Alternatively, the small-scale spatial and temporal variability in the physical-chemical composition of the environment (Chevaldonné *et al.*, 1991) might have caused the gastropod population to show local variations in bacterial symbiont stock. Both options have also been considered by Ohta *et al.* (1988), who postulated that methanotrophic symbionts were present only in low abundance (up to 10% of the symbionts). They also discussed the possibility that the methanotrophic bacteria might not be present in all members of the gastropod population, depending on the ambient conditions. Endow and Ohta (1989b) suggested that the putative methanotrophic symbionts of *A. hessleri*, as well as the prevailing thiotrophic type, might have been acquired

"horizontally" from the outside. This would relate the symbiosis to environmental conditions, *i.e.*, the presence of the symbionts in the environment. From an adaptive perspective, it would lend the host a favorable flexibility to cope with the high spatial and temporal variability of the vent ecosystem and might explain why *A. hessleri* and *I. nautili* are mixotrophs that have retained a functional, though reduced, digestive tract (Bouchet and Warén, 1991; Warén and Bouchet, 1993; Desbruyères *et al.*, 1994). It could then be assumed that the composition of thiotrophic and methanotrophic bacteria reflects the composition of the reduced compounds released from the vents (Distel *et al.*, 1995). This variability would also explain the inconsistency in our measurements of S° in *I. nautili*, which varied by a factor of 8. These considerations imply that assessment of one or two types of symbionts might depend on fortuitous individual temporal or local conditions. Physiological assessment through diagnostic enzymes might be problematical because low concentrations can escape detection (Endow and Ohta, 1989b). Hence, one probably has to apply molecular techniques to reliably demonstrate the presence or absence of different symbiont types. The occurrence of more than one type of symbiotic bacteria in one host has been shown to exist in several populations of the mytilid bivalves (Childress *et al.*, 1986; Fisher *et al.*, 1987; Cavanaugh *et al.*, 1987; Fisher *et al.*, 1993; Distel *et al.*, 1995) and in gutless oligochaetes (ultrastructural evidence: Giere *et al.*, 1995; molecular evidence: Dubilier *et al.*, unpubl. data).

Genetic differences between specimens from the Manus and the North Fiji Basin might also develop in response to the spatial and temporal instability of hydrothermal vents. Genetic separation in populations of neighboring vent fields has been shown to occur in *Alviniconcha hessleri* (Denis *et al.*, 1993) and in the bivalves *Calyptogenia* spp. (Vrijenhoek *et al.*, 1994) and *Bathymodiolus* spp. (Craddock *et al.*, 1995).

Ecological considerations and evolutionary aspects

It is commonly argued that there is an evolutionary trend from extra- to intracellular symbioses (Smith, 1979; Southward, 1986; Craddock *et al.*, 1995; Giere, 1996; Ott, 1996). In gastropods, the first step might be represented by *Lepetodrilus fucensis* from the northeastern Pacific. In all sections of this vent limpet, bacteria originating from the dense colonies of bacteria on the epithelial surface of the gill have been observed to become endocytosed and digested in lysosome-like organelles (De Burgh and Singla, 1984). This has been interpreted as a routine process that occurs in addition to the normal grazing on the ambient bacterial population. In *Alviniconcha hessleri* and *Ifremeria nautili* the symbiotic bacteria are enclosed

in vacuoles, but still appear to have some contact with the outside. This would represent an intermediate step in a morphological series. The most advanced step, complete incorporation of symbionts into bacteriocytes, is common in many bivalve molluscs, but has not yet been described from symbiont-harboring gastropods.

The bacterial symbioses in *A. hessleri* and *I. nautilei* are structurally very similar, and the two hosts often settle close together in hydrothermal fields. Taxonomically, however, the snails have been assigned to different gastropod genera. This taxonomic difference might reflect a slight ecological or physiological niche diversification. Usually, *A. hessleri* has been found a little closer to the hydrothermal outlets than *I. nautilei* (Bouchet and Warén, 1991; Galchenko *et al.*, 1992; Warén and Bouchet, 1993; Desbruyères *et al.*, 1994). It seems possible that this pattern is temperature dominated and mirrors specific differences in temperature preference. In the North Fiji Basin, Chevaldonné *et al.* (1991) obtained data on temperature gradients and the distribution of the gastropod colonies. But the complex temperature pattern seems to change locally very much and, for the area investigated here, it is not possible to relate the distribution pattern of the two gastropods to the temperature gradients around the hydrothermal outlets.

In molluscs, symbiosis obviously developed several times independently (Distel *et al.*, 1994). The gills of bivalves and gastropods harbor different types of bacteria, sometimes even in an individual host. The present study indicates that separate populations of the same species may differ in their bacterial partners. An intraspecific symbiotic variability between bacterial groups (methanotrophs and thioautotrophs) may have contributed to the success of these gastropods in the unstable and ecologically diverse ecosystems of Pacific hydrothermal vents (Van Dover, 1990). The reasons for their distributional restriction to the Southwest Pacific remain unclear, but a more detailed picture of the symbiotic variability and ecological versatility may help to clarify it.

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Limb Regeneration in the Eye Sockets of Crabs

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Abstract. The eyestalks of crabs were removed and various tissues of the limbs were autotransplanted into the empty eye sockets to study the capacity of the limb tissue to regenerate in a heterotopic site. Autotransplantation of walking leg tissues into the eye sockets was able to regenerate complete walking legs in the new site. Autotransplantation of tissues of claw digit (dactyl and pollex) or more proximal claw segments (ischium and merus/carpus joint) could regenerate complete claws in the eye sockets. If the autotransplant of claw tissue was contralateral, claws could regenerate with host-site handedness. Sham operations or autotransplantation of frozen claw tissue did not induce regeneration in the eye sockets. These results demonstrate that complete crab claws can regenerate from the eye sockets by autotransplantation of live limb tissue and that the regeneration is not due to the traumatic effect of transplantation.

The structure of the limbs regenerated in the eye sockets was determined by the source of the transplanted tissue. Complete claws resulted from autotransplantation of the tissues of the most distal claw segments (claw digits), and the most distal claw segments regenerated first, followed by the proximal claw segments in subsequent molts. Thus tissue from distal portions of crab claw can regenerate proximal portions of the claw in the eye sockets. Such a mode of regeneration is not consistent with the distalization rule of the polar coordinate model, which proposes that distal portions of the limb cannot regenerate proximal portions and that the direction of limb regeneration is always from proximal to distal.

Introduction

Our previous study (Kao and Chang, 1996) showed that autotransplantation of claw tissues into the autotomized

stumps of crab walking legs can induce the stumps to regenerate claws or chimeras of claw and walking leg. The percentage of the claw or clawlike structures regenerated from the walking leg stumps was higher when the autotransplant consisted of a combination of claw tissues than when only a single type of tissue was used. With contralateral autotransplantation of claw tissues into the autotomized stumps of the walking leg, the stumps can regenerate claws with host-site handedness.

To further study whether claw tissues—singly or in combination—can regenerate complete claws in a heterotopic site that lacks a regenerating limb field, we autotransplanted claw tissues into the carapace at sites from which the eyestalks had been removed (these sites are hereafter called eye sockets). Crabs can regenerate their autotomized limbs but not their eyestalks (reviewed by Hopkins, 1988). There are no reports of crabs found in nature having limbs in the place of the eyestalks. The eye sockets thus provided an *in vivo* environment that was isolated from the tissue of the autotomized limb stumps.

In this study, we autotransplanted either single claw tissue or different combinations of claw tissues of the distal or more proximal claw segments into the eye sockets to determine whether there was any differential regenerative capacity. It has been proposed that the distal portions of the limbs cannot regenerate the proximal portions of the limbs (distalization rule of the polar coordinate model; Bryant *et al.*, 1981) and that different sizes or combinations of tissues can determine the regenerative capacity of hydra (Shimizu *et al.*, 1993) or the imaginal discs of *Drosophila* (Kauffman and Ling, 1981). In addition, we contralaterally autotransplanted limb tissues into the eye sockets to study whether the handedness of the regenerated limbs would be changed by the host site.

Materials and Methods

Animals and dissection

Two species of crabs were used: *Cancer gracilis* (range of carapace width = 6.9–27.9 mm, mean $\pm$ SD = 12.08

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± 3.49 mm, $n = 211$) and *C. jordani* (range of carapace width = 8.2–17.2 mm, mean $\pm$ SD = 11.55 ± 2.67 mm, $n = 12$). Specimens were collected subtidally with a hand net in Bodega Harbor, California. The crabs were kept in individual compartments to prevent cannibalism (Conklin and Chang, 1993) and were fed brine shrimp, shrimp meat, or fish every other day. Crabs were acclimated in the laboratory for at least one molt prior to experimentation. Only postmolt animals (less than 3 days after molt) were used.

Animals were forced to autotomize one limb—either a claw or the fourth walking leg. (Crab limbs are described and illustrated in Kao and Chang [1996].) Autotomy was achieved by crushing the manus or carpus segment of the limb with a pair of forceps. For donor tissues, the dactyl, pollex, ischium, and merus/carpus joint of the autotomized claw were used. In other experiments, the dactyl, ischium, and merus/carpus joint of the autotomized walking leg were used. These segments were cut off with a pair of fine scissors and placed into a depression slide with a drop of crab saline (440 mM NaCl; 11.3 mM KCl; 13.3 mM CaCl_2 ; 26 mM MgCl_2 ; 23 mM Na_2SO_4 ; 10 mM HEPES, pH 7.4 with NaOH; 100 units/ml penicillin; 0.1 mg/ml streptomycin; Cooke *et al.*, 1989). The slides were placed under a dissecting microscope and fine forceps were used to carefully pull intact tissues (including epidermis and muscle) out of the exoskeleton of each segment.

Removal of eyestalks and autotransplantation of limb tissues

One eyestalk of each crab (*C. gracilis*, $n = 211$; *C. jordani*, $n = 12$) was extirpated as proximally as possible with a pair of forceps under a dissecting microscope. This procedure left an opening at the site of entry of the eyestalk into the carapace (eye socket). Immediately after eyestalk removal, limb tissues were autotransplanted or sham operations were performed. The animals were then returned to seawater.

Autotransplantation consisted of pushing limb tissue into the eye socket, using the blunt end of an insect pin, until the tissue could no longer be seen with a dissecting microscope. Transplants were of two types: ipsilateral, in which claw tissue was removed from one side of the crab body and placed into the eye socket on the same side (e.g., left claw tissues autotransplanted to the left eye socket of the same animal); and contralateral, in which the claw tissues were placed in the eye socket on the opposite side of the crab (e.g., left claw tissues autotransplanted into the right eye socket of the same animal).

To examine the regenerative ability of different claw segments, single claw digits (dactyl or pollex), both claw digits (dactyl and pollex), or proximal segments of the

claw (ischium + merus/carpus joint) were autotransplanted into eye sockets. To see whether the host site could regulate the morphology of the regenerated claw, different claw tissues (dactyl or pollex) or combinations of claw tissues (dactyl + pollex, ischium + merus/carpus joint, or dactyl + pollex + ischium) were contralaterally autotransplanted into eye sockets. To see whether dead tissues had the ability to induce regeneration in the eye socket, frozen claw tissues were autotransplanted into eye sockets.

To determine whether walking legs can regenerate from eye sockets, tissues from the dactyl, ischium, and the merus/carpus joint of the fourth walking legs were contralaterally autotransplanted into eye sockets. Control sham operations were conducted by inserting an insect pin three times into an eye socket. The transplantations were considered failures if no limb or limblike structure regenerated from the eye sockets after the fourth postoperative molt.

Characteristics of axes and handedness in C. gracilis

For the convenience of description, three axes of claws are defined. For the proximodistal axis, the proximal segments are the segments closest to the crab body. The distal segments are the segments farthest away from the body. Thus, the coxa and ischium are proximal segments of the claw; the dactyl and pollex are the distal "digits" of a claw. For the anteroposterior axis, the dactyl is the anterior movable "digit" of a claw and the pollex is the posterior fixed "digit" of a claw. For the dorsoventral axis, the dorsal surface of the claw manus has four ridges (carinae, Fig. 1A) along the proximodistal direction. The ventral surface of the claw is relatively smooth and lacks carinae (Fig. 1B). The dorsal surface of the walking leg has small granulosa tubercles and darker purple coloration (Fig. 1C), and the ventral surface of the walking leg is relatively smooth and lighter in color (Fig. 1D). The handedness of a claw can be easily distinguished by bending it. When the claw dactyl is oriented above the claw pollex, the right claw bends to the left and the left claw bends to the right. Handedness of the fourth walking leg can be identified by combinations of bending and axis characteristics. When viewed dorsally, the fourth right walking leg bends in a clockwise direction and the fourth left walking leg bends counterclockwise.

Results

Autotransplantation of limb tissues into eye sockets of C. gracilis

After autotransplantation of crab limb (claw or walking leg) tissues into crab eye sockets, limbs or limblike structures regenerated from some eye sockets (22.1% of sur-

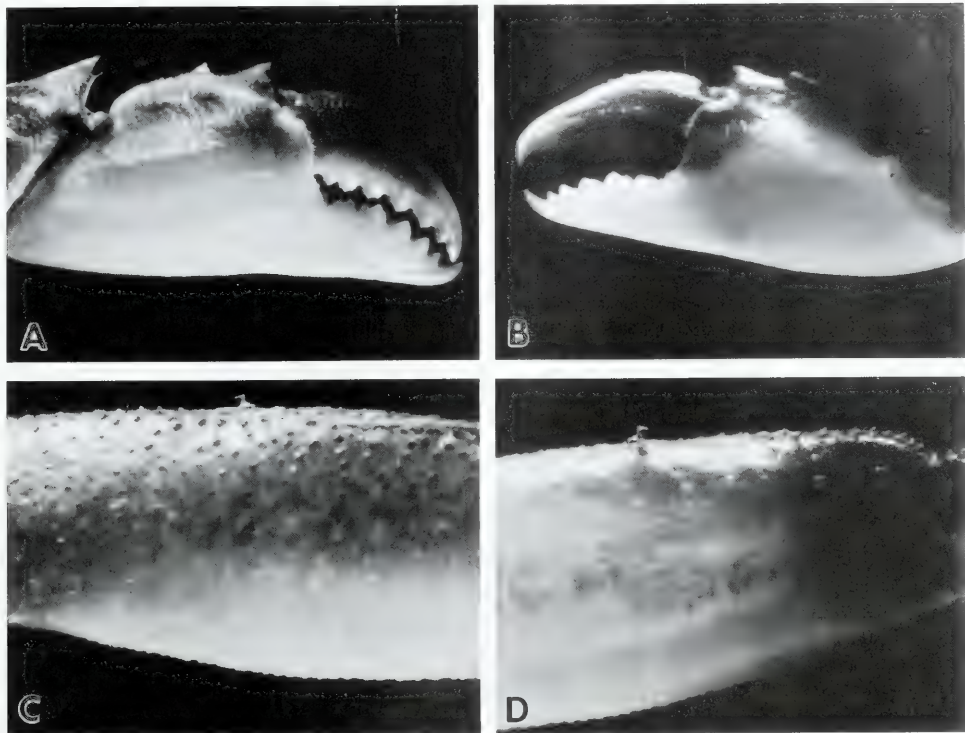


Figure 1. Characteristics of *Cancer gracilis* limbs. (A) Dorsal surface of claw manus has four ridges (carinae). (B) Ventral surface of claw manus does not have ridges. (C) Dorsal surface of the fourth walking leg has granulosa tubercles and darker pigmentation. (D) Ventral surface of the fourth walking leg does not have granulosa tubercles and has lighter pigmentation.

viving crabs that received live limb tissues, $n = 104$; Table I, Fig. 2A–I). According to our definitions, “regenerated” limbs contained at least two segments, and their handedness was recognizable; “limblike” regenerates consisted of one or more segments, but their handedness could not be recognized. Autotransplantation of claw tissues resulted in regeneration of claws or clawlike structures from eye sockets (20.6% of surviving crabs that received live claw tissues; Fig. 2A–H). Autotransplantation of fourth walking leg tissues resulted in regeneration of walking legs or leglike structures from the eye sockets (33.3% of the surviving crabs; Fig. 2I). Sham-operated crabs or autotransplantation of frozen claw tissues into eye sockets did not regenerate any limb or limblike structure from the sockets (Table I).

The number of crab molt cycles needed to regenerate these structures and the number of claw segments regener-

ated per molt were variable among crabs. Regeneration of claws with complete segments (Figs. 2A, B, 3A) usually took two to four postoperative molts. In most cases, crabs regenerated one or two distal segments after the second postoperative molt (Fig. 2A, B) and the rest of the proximal segments after the third or fourth postoperative molts. Some crabs, however, regenerated complete claws after the second postoperative molt (Fig. 3A). Both sham-operated and autotransplanted crabs regenerated normal limbs from their autotomized stumps after the first postoperative molt regardless of whether limbs (or limblike structures) were regenerating in their eye sockets. The duration of the first postoperative molt interval of the sham-operated ($22.44 \pm 1.08d$, $n = 18$) and experimental ($20.8 \pm 0.56d$, $n = 91$) animals was variable among individuals of similar sizes and not statistically different between the two groups ($P = 0.094$).

Table 1

Summary of limb tissue autotransplantation in the eye socket of
Cancer gracilis (carapace width = 12.05 ± 3.40 mm)

Donor tissue	Host eye	Trials (No.)	Survival (No.)	* Type of regenerate	
				Limblike	Limb
Claw					
dactyl	ipsi	15	6	0	0
dactyl	contra	17	8	1	0
pollex	ipsi	15	8	2	0
pollex	contra	10	3	1	0
dactyl + pollex	ipsi	19	13	2	2
dactyl + pollex	contra	23	15	1	1
frozen dactyl + pollex	ipsi	12	9	0	0
dactyl + pollex + ischium	contra	20	15	1	3
ischium + joint	ipsi	23	14	1	1
ischium + joint	contra	13	10	1	2
Walking leg					
dactyl + ischium + joint	contra	19	12	3	1
Sham operation	ipsi	25	18	0	0
Total		211	131	13	10

* Limblike: a structure that resembles a claw or walking leg and in which handedness cannot be recognized; limb: a claw or walking leg in which handedness can be recognized.

Both distal and proximal limb tissues can regenerate complete limbs

Autotransplantation of a combination of distal and proximal claw segments (dactyl + pollex + ischium) into eye sockets regenerated claws or clawlike structures in four cases (26.7% of surviving crabs, $n = 15$). Among them, three were claws (Fig. 2A, B) and one was a tapered projection. The claws had both claw digits and at least two segments. In addition, the digits had toothlike structures. The tapered projection had neither a joint nor any teeth. Its size increased after the crab molted, but the shape had not changed after four postoperative molts.

Autotransplantation of proximal segments of claws (ischium + merus/carpus joint) or distal parts of claws (dactyl + pollex) regenerated claws or clawlike structures (11 cases, 21.2% of surviving crabs, $n = 52$). Among them, six were claws (Fig. 2C) and five were clawlike structures (Fig. 2D–F). The clawlike structures included Y-shaped (Fig. 2D), pollexlike structures (Fig. 2E), and a structure similar to the manus and carpus segments of the claw (Fig. 2F). The Y-shaped limb (Fig. 2D) resulted from contralateral autotransplantation of dactyl and pollex tissues of the claw. The pollexlike structure (Fig. 2E) regenerated from lateral autotransplantation of dactyl and pollex tissues of the claw. It had two rows of teeth but no

pigmentation. The structure similar to the claw manus and carpus segments (Fig. 2F) resulted from autotransplantation of the ischium and merus/carpus joint of the claw.

Autotransplantation of single digits of claws (dactyl or pollex) regenerated tapered projections, characterized by the lack of a joint, in four cases (16% of surviving crabs, $n = 25$; Fig. 2G, H). These crabs failed to regenerate claws after four postoperative molts. Autotransplantation of fourth walking leg tissues (dactyl + ischium + merus/carpus joint) regenerated one complete walking leg (Fig. 2I) and three incomplete walking legs that contained only distal segments (dactyl or dactyl + manus) of the fourth walking leg.

Two modes of regeneration in eye sockets after autotransplantation of crab limb tissues

Two modes of regeneration were observed. One mode—regeneration of a complete claw following ecdysis—can occur in autotransplantation of both digits of the claw, the proximal segments of claws (ischium + merus/carpus joint), or a combination of distal and proximal segments of claws (dactyl + pollex + ischium). In this form of regeneration, a budlike structure emerged from the eye socket before ecdysis, but no limb or limblike structures were observed at that time. After ecdysis, a complete claw regenerated from the eye socket.

The second mode of regeneration occurred in autotransplantation of a combination of distal and proximal claw tissues (dactyl + pollex + ischium) or of both claw digits (dactyl + pollex), but not in autotransplantation of the proximal claw segments (ischium + merus/carpus joint). In this mode, distal segments (dactyl or dactyl + pollex; Fig. 2C) regenerated first, followed by the appearance of proximal segments after subsequent molts. For the claw shown in Figure 2B, for example, we observed a dactyl emerge from the eye socket after the second postoperative molt. Both the dactyl and pollex were present in the eye socket after the third postoperative molt; a complete claw regenerated from the eye socket after the fourth postoperative molt. To confirm that only distal claw segments initially regenerated in the eye sockets, some crabs were dissected immediately after the distal segments first emerged from the eye sockets, and crab exuviae were examined at successive postoperative molts. We found no claw segments hidden inside the carapace that could not be observed from the exterior under a dissecting microscope, and we confirmed that those initially regenerated claw segments contained only distal claw segments. Those crabs contained either a single claw digit (dactyl), both claw digits (dactyl and pollex), or claw digits and a partial manus segment (Fig. 2C).

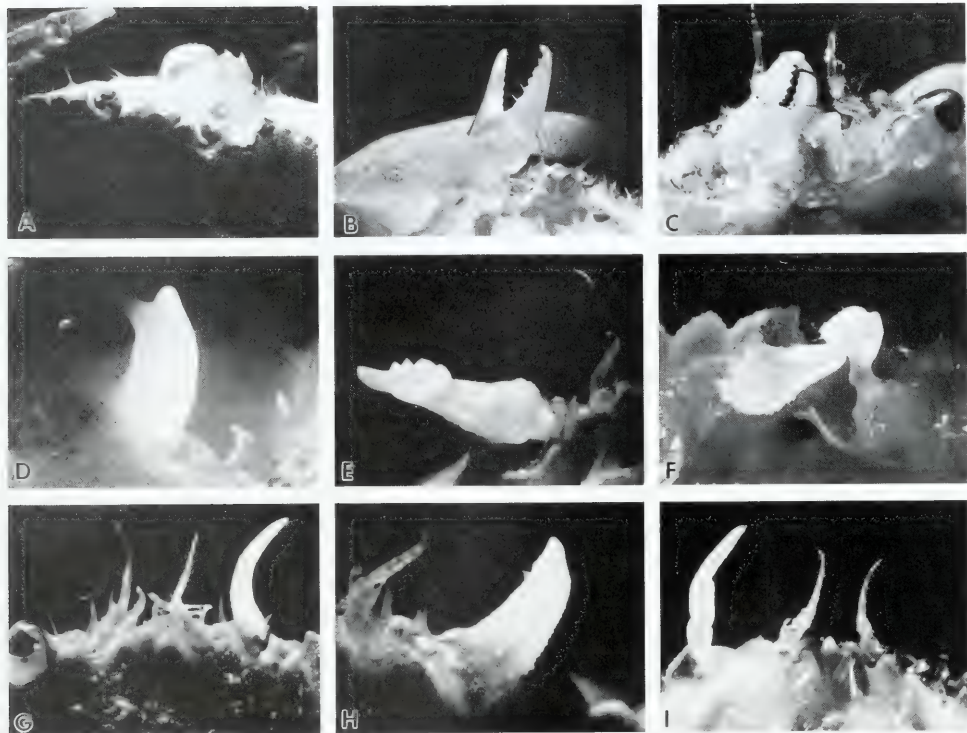


Figure 2. Limb or limblike structures regenerated from *Cancer gracilis* eye sockets. (A) Complete claw regenerated from the eye socket after the fourth postoperative molt (M4). (B) Complete claw with a mixture of donor and host-site handedness after M4. (C) Claw with dactyl, pollex, and partial manus segments after the second postoperative molt (M2). (D) Y-shaped limb after M2. (E) Pollexlike structure after the third postoperative molt. (F) Clawlike structure with manus and carpus segments after M2. (G, H) Distally tapered regenerates after M4. (I) Complete walking leg after M2.

Handedness of regenerated limbs in C. gracilis

Contralateral autotransplantation of claw tissues into eye sockets regenerated six claws with recognizable handedness. Among them, two claws had host-site handedness (Fig. 2A), three claws had donor-tissue handedness, and one claw had a mixture of host and donor handedness (Fig. 2B). The two claws with host-site handedness resulted from contralateral autotransplantation of a combination of distal and proximal claw tissues (dactyl + pollex + ischium; Fig. 2A), or proximal claw tissues (ischium + manus/carpus joint). The claw with a mixture of host and donor handedness resulted from autotransplantation of a combination of distal and proximal tissues (dactyl + pollex + ischium; Fig. 2B). It had donor handedness at its distal parts and host handedness at the proximal parts

of the claw. The claw digits regenerated first, and the proximal segments regenerated during subsequent molts. Ipsilateral autotransplantation of claw tissues regenerated three claws with recognizable handedness. These three claws retained the donor (same as the host-site) handedness (Fig. 2C). Contralateral autotransplantation of the fourth walking leg tissues into eye sockets regenerated one walking leg with recognizable, donor-tissue handedness (Fig. 2I).

Autotransplantation of claw tissues into eye sockets in C. jordani

Contralateral autotransplantation of *C. jordani* claw tissues into eye sockets regenerated one complete and one incomplete claw (Table II). The complete claw (Fig. 3A)

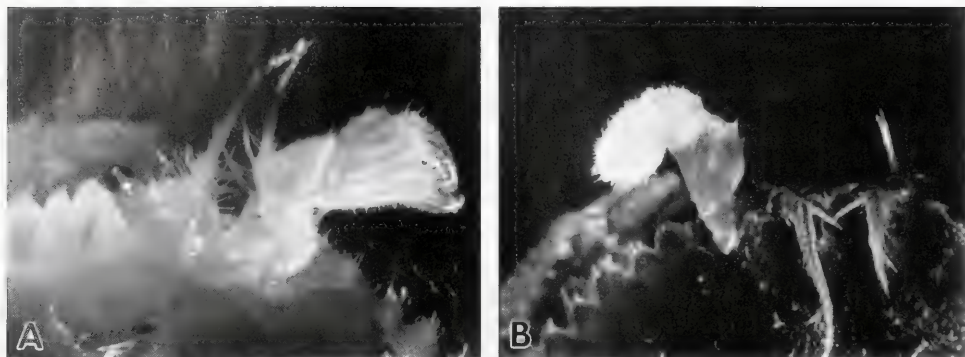


Figure 3. Claw regenerated from *Cancer jordanii* eye sockets. (A) Complete claw regenerated from the eye socket after the second postoperative molt (M2). (B) Incomplete claw with all segments proximal to the claw digits regenerated after M2.

resulted from contralateral autotransplantation of both claw digits and had host-site handedness. Like an authentic claw, it contained five segments. The incomplete claw (Fig. 3B) resulted from contralateral autotransplantation of the claw pollex and had donor-tissue handedness. It had a fork-shaped structure at the distal tip and four normal proximal segments. The fork-shaped structure was regenerated first, and the proximal segments were regenerated in subsequent molts. All sham-operated animals failed to regenerate limbs in eye sockets.

Discussion

Kim and Stocum (1986) autografted anterior or posterior halves of axolotl forearms to eye sockets and amputated them distally 7 days later. The transplants could not regenerate proximal structures of the limbs. They either regressed until a small fragment of the radius remained in the orbit or regenerated no more than two digits distally. Pecorino *et al.* (1996) transplanted the hindlimb distal blastema to the forelimb proximal stumps of newt limbs. The proximal part of the regenerate was mostly generated by the stump, and the transplanted cells made only a

minor contribution. These results, together with older studies (Dent, 1954; Butler, 1955), indicate that amphibian limbs can regenerate distally but not proximally, and they are consistent with the distalization rule of the polar coordinate model (Bryant *et al.*, 1981).

When we autotransplanted claw tissues into the eye sockets, the transplanted tissues were completely inside the crab body. This is in contrast to the transplantation methods used in amphibians, in which only the most proximal portions of the limbs or regeneration blastemas were inserted into the body. Our results showed that complete claws can regenerate from the eye sockets by autotransplantation of tissues of either distal (dactyl and pollex) or more proximal claw segments. Even autotransplantation of a single claw digit (pollex) resulted in regeneration of a claw with complete proximal segments (ischium, merus, carpus, and manus) but with incomplete distal segments (without a dactyl or pollex, Fig. 3B). In addition, we also observed that distal claw segments can regenerate initially, followed by regeneration of proximal segments after subsequent molts. These results demonstrate that complete claws can regenerate from crab eye sockets by autotransplantation of tissues of the distal claw segment and that claw regeneration in the eye sockets can proceed in a distal to proximal direction. Such a mode of regeneration is inconsistent with the results observed in amphibians and with the distalization rule of the polar coordinate model (Bryant *et al.*, 1981). This inconsistency may be due to differences in the grafting methods or species used.

Although antenna-like structures or antennules growing from eye sockets have been observed in both wild and experimental decapods (Bateson, 1894; Maynard, 1965; Nevin and Malecha, 1991), this phenomenon has not been

Table II

Summary of limb tissue autotransplantation in the eye socket of *Cancer jordanii* (carapace width = 10.79 ± 4.05 mm)

Donor tissue	Host eye	Trials (No.)	Survival (No.)	Claw
pollex	contra	1	1	1
dactyl + pollex	contra	8	4	1
Sham operation	contra	3	2	1

reported for crabs. We also did not observe such structures in our experimental or sham-operated crabs. Some animals regenerated tapered projections (Fig. 2G, H) or structures different from the original grafted tissues in the eye sockets (Fig. 2E, F). They were, however, neither an antennule nor an antenna, because both appendages had multiple segments. Autotransplantation of a single claw digit into eye sockets resulted in a low percentage of regenerates in the eye socket, and all the regenerates were tapered or distally incomplete. We believe that the claw tissues in the eye sockets underwent regression and that regeneration of a complete distal structure is dependent upon a complete proximal claw structure. This obeys the complete circle rule of the polar coordinate model (French *et al.*, 1976; Bryant *et al.*, 1981).

Autotransplantation of a claw pollex into an eye socket regenerated a claw with incomplete distal segments but complete proximal segments. A Y-shaped structure initially emerged from the eye socket after the first postoperative molt, and a claw with complete proximal segments but incomplete distal segments regenerated from the eye socket after the second postoperative molt. This result suggests that claw proximalization, unlike distalization, need not have a complete structure at the distal tip. Limb tissues grafted into eye sockets did not regenerate eye structures. Instead, only limb structures regenerated from eye sockets. Our results showed that limb tissues are not pluripotent; their fates have been determined to regenerate limbs even in the eye sockets.

We observed that claw regeneration in the eye sockets can proceed from the distal to the proximal parts of the claws (Fig. 2C, J). This process can occur over four molt cycles. Examination of dissected animals and crab exuviae revealed that this mode of regeneration is produced by continuously generating blastemas at the proximal ends of the previous regenerates. The proximal ends of blastemas and future regenerated claws were not suspended in the crab body. Instead, they fused with tendons of eyestalks (Fig. 3A), remnants of eyestalks (Fig. 2F), or the carapace near the eye sockets (Fig. 2C). This explains why they were not shed with the old exoskeleton during molt. We observed that although the proximal segments of the regenerated claws were capable of movement, the dactyl was not. Thus they were not fully functional claws.

In our experiments, a total of 12 limbs (11 claws and 1 walking leg) with recognizable handedness regenerated from the crab eye sockets. Among them, 8 limbs (7 claws and 1 walking leg) retained the donor-tissue handedness regardless of whether the autotransplantation of claw tissues was ipsilateral or contralateral. Three regenerated claws changed from donor-tissue to host-site handedness, and one regenerated claw had a mixture of donor and host handedness following contralateral autotransplantation of

claw tissues into eye sockets. The mechanism for change of handedness of crab claws is unknown, but our observations suggest that the axis of grafted tissues in the eye socket has been changed.

Handedness of limbs is determined by the dorsoventral, proximodistal, and anteroposterior axes. Left and right hands have the same dorsoventral and proximodistal axes but are opposite in the anteroposterior axis. One explanation for the change of claw handedness is that the grafted claw tissues in the eye sockets have been reorganized. It is known that limb regeneration does not take place by direct outgrowth but by the production of undifferentiated blastema cells. The blastema cells are derived from dedifferentiation of stump tissues (Adiyodi, 1972; Stocum, 1991; Tsonis *et al.*, 1995). Since we grafted a piece or several pieces of claw tissues into eye sockets, the dedifferentiation process might occur in all pieces of the transplants. Dedifferentiation of limb tissue might generate a new limb primordia in which the axes are undetermined. With a complete dedifferentiation of claw tissues in eye sockets, the handedness of claws might be determined by the host site. Without a complete dedifferentiation of claw tissues in eye sockets, the handedness of the regenerating claw might be inherited from the original donor tissues. A mixture of donor and host handedness may be due to the influence of both the donor tissues and the host site. Alternatively, it is possible that the handedness of the claws regenerating in the eye sockets might be determined by a random process unrelated to the handedness of the grafted tissues or host sites.

Acknowledgments

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Claw Transformation and Regeneration in Adult Snapping Shrimp: Test of the Inhibition Hypothesis for Maintaining Bilateral Asymmetry

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Abstract. In the paired asymmetric claws of adult snapping shrimp, *Alpheus heterochelis*, the minor, or pincer, claw may transform into a major, or snapper, claw if the existing snapper claw is damaged or lost, implying that an intact snapper claw normally inhibits the contralateral pincer claw from advancing to a snapper. We find that the pincer-to-snapper advancement in external form occurs almost immediately after the snapper is lost even as late as the premolt stage. The transforming claw in turn inhibits the newly regenerating pincer claw from becoming a snapper, but if the dactyl of the transforming claw is cut, then snapper-based inhibition is removed and the contralateral claw may regenerate as a snapper, resulting in shrimp with paired snapper claws. However, damaging an established snapper claw will not allow another snapper claw to regenerate at the pincer site, implying that less inhibition is required to restrict a newly regenerating claw to a pincer than to arrest an existing pincer claw. Inhibition may be manifested largely in terms of quantity of innervation. Hence the greater innervation of the snapper side over the pincer side would inhibit the pincer side, accounting for the regeneration of paired claws in their previous configuration following loss of both claws. Loss of the paired claws in two consecutive molts retards their development so that both claws often appear as pincers, but in succeeding molts one usually differentiates into a snapper and bilateral asymmetry is restored. In contrast, shrimp with paired snapper claws retain this configuration over several molts unless one or both of the claws are lost; in that case, regeneration restores bilateral asymmetry. Thus, bilateral asymmetry of the paired claws of adult

shrimp is governed by a strong intrinsic lateralizing mechanism in which the snapper claw inhibits the pincer from advancing to another snapper.

Introduction

Among crustaceans, bilateral asymmetry of the first pair of chelipeds is common; one of the paired claws is more enlarged and elaborate (major claw) than the other (minor claw). In snapping shrimps of the family Alpheidae, the major, or snapper, claw is almost as large as the abdomen and has a hammer on the moveable dactyl that fits into a reciprocal socket on the fixed pollex (Fig. 1A) (Przibram, 1901). The closing action of the hammer into the socket is made with such tremendous force that it is accompanied by a loud popping sound and a jet-expulsion of water, both of which are used in agonistic encounters (Hazlett and Winn, 1962; Ritzmann, 1974) or in crushing bivalve shells (McLaughlin, 1982). The minor, or pincer, claw is much smaller and is used in burrowing and feeding.

An unusual feature of claw bilateral asymmetry in snapping shrimp is the ability to reverse its configuration. Loss of the snapper early in an intermolt results in the transformation of the pincer to a snapper and the regeneration of a new pincer at the snapper site at the next molt (Przibram, 1901; Wilson, 1903). In addition to loss of the snapper claw, less drastic measures such as its denervation (Mellon and Stephens, 1978), dactylotomy (Read and Govind, 1997), or closer muscle tenotomy (Govind *et al.*, 1988) are also sufficient to trigger transformation of the pincer into a snapper. Because the existing snapper claws repair themselves, such procedures produce shrimp that possess paired snapper claws. Since these manipulations that induce pincer-to-snapper transformation involve

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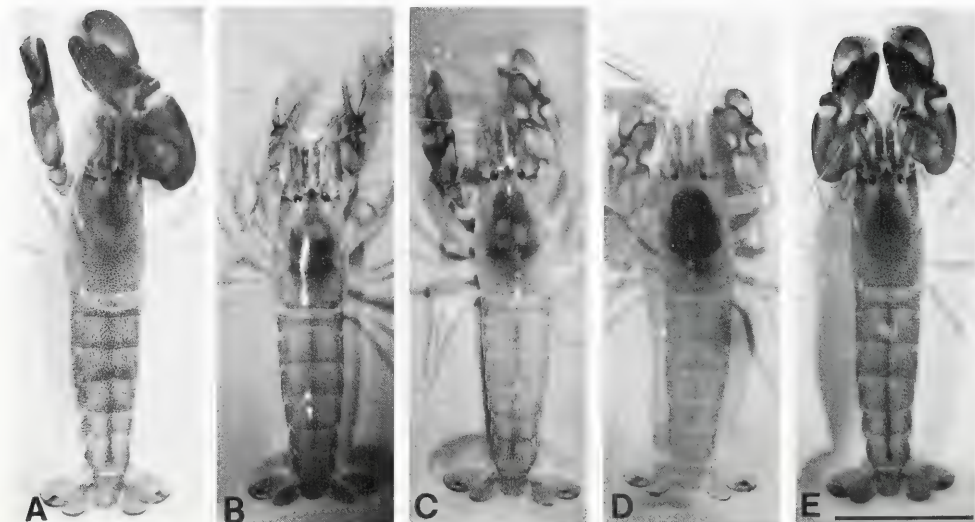


Figure 1. Adult snapping shrimp showing different configurations of their paired claws. (A) Pristine asymmetric configuration in which the snapper (right claw) is extremely hypertrophied, with a pronounced hammer and socket, and the pincer (left claw) is small, slender, and lacks the snapping apparatus. (B) Newly regenerated paired pincer claws which are smaller than their pristine counterparts. (C) Paired snapper claws with newly regenerated snapper (right claw) and dactyl-less transformed pincer (left claw). (D) Newly regenerated paired snapper claws which are much smaller and not as highly differentiated as their pristine counterparts. (E) Pristine paired snapper claws in which the paired claws are similar in size and differentiation. Scale bar 10 mm. $\times 2.5$

damage to the nervous system of the snapper claw, it is likely that the transformation results from loss of the neural inhibition by which the snapper claw prevents the pincer claw from completing its development to a snapper (Wilson, 1903). Because regenerating claws in adult shrimp pass through a distinct pincerlike stage before differentiating into a snapper claw (Wilson, 1903; Darby, 1934; Read and Govind, 1997), we have devised a scheme in which loss of the snapper claw removes its inhibition on the pincer, which then advances to a snapper and in turn inhibits the newly regenerating claw to a pincer.

Snapper-based inhibition of the pincer claw can also explain why loss of the pincer claw in adult shrimp results in the regeneration of another pincer (Wilson, 1903). To explain the fact that simultaneous loss of both claws results in claw regeneration in the same configuration, we would have to assume that snapper-based inhibition prevails even in the absence of claws. Thus, no matter which claw is lost, inhibition by the snapper claw (or its site) on the pincer claw (or its site) ensures regeneration of bilateral asymmetry. A simple and economical scheme for explaining how bilateral asymmetry is maintained in the face of claw loss and regeneration in adult snapping shrimp is that of Wilson (1903), who regarded the pincer

as an arrested snapper. We have adopted this scheme with the assumption that the inhibitory signal has a neural basis. That assumption largely rests on the fact that cutting the nerve in the snapper claw is enough to trigger transformation of the pincer to a snapper. The inhibitory signal may therefore be easily manipulated with minor surgery of the claws. Here we describe experimental manipulations designed to explore some ramifications of the inhibition hypothesis for claw bilateral asymmetry in adult snapping shrimp. Our findings support the existence of a lateralizing mechanism that is based on inhibition from the snapper claw or from its site and can switch from one side to the other.

Materials and Methods

Adult snapping shrimp, *Alpheus heterochelis*, of both sexes were collected at low tides off the coast of Beaufort, North Carolina, and transported to Scarborough, Ontario, where they were held in the laboratory at room temperature, 23°C. The shrimp were housed in 25-liter glass aquaria partitioned into 12 compartments with plastic screening (Young *et al.*, 1994)—one shrimp per compartment. They were fed at 2–3 day intervals with a specially

prepared diet consisting of a mixture of fish, beef heart, carrots, and commercial trout chow. Under these conditions the shrimps had an intermolt period between 19 and 26 days, for an average of 23 days. A detailed molt history was kept for each animal, and in most cases experimental manipulations were carried out 1 or 2 days after ecdysis.

All animals were allowed to molt twice before being selected for study in order to ensure that the claws were fully differentiated (Read and Govind, 1991). Several types of experimental manipulations were made. The simplest one was to induce the animal to autotomize its claw after ecdysis by gently pinching the limb with forceps just distal to the autotomy plane. For more complex manipulations, the shrimp were first anesthetized by cooling; then, either the dactyl of the snapper claw was cut close to its attachment to the claw so that most of it was removed, or the nerve in the snapper claw was sectioned. The latter was accomplished by cutting a small flap of cuticle in the ventral side of the merus and pulling nerve 2 (the larger of two) so that it broke more proximally at the autotomy plane. After a substantial length of nerve 2 had been removed, the cuticular flap was replaced and the shrimp were treated with a wide-spectrum antibiotic (Paragon) for the next two days. Limb immobilization was achieved by anesthetizing the shrimp, then applying cyanoacrylate glue to the thoroughly dried converging edges of the propus and dactyl. Experimental manipulations were usually performed 1–2 days after a molt, and the experimental shrimp were observed over the next two to three intermolts.

Fiber composition of the claw closer muscle was determined by obtaining frozen cross-sections of the claws and staining these histochemically so that standard techniques for detecting myofibrillar ATPase activity (Ogonowski and Lang, 1979) could be applied.

To evaluate the degree of transformation of pincer to snapper claw, a transformation index, based on five morphological features, each representing a graded measure of a unique snapper characteristic, was devised. The first feature, based upon the presence and development of the plunger and socket, was given a weighting of 4 points and determined by comparing the transforming claw with a standard developmental series similar to that in Figure 2. For example, a transforming claw with no plunger or socket (similar to a pristine pincer) would be assigned 0 points, while one with a plunger and socket (similar to a pristine snapper) would be allotted 4 points. The second feature, stoutness, was weighed at 3 points and based upon the ratio of propus length to width. The smaller the ratio, the stouter and more snapper-like the claw, and hence the more points allocated. The last three features were weighted at 1 point each and based upon the presence and development or abundance of the transverse groove, tubercles, and plumose setae (Read and Govind, 1991). The maximum possible transformation index score

was 10, the equivalent of a pristine snapper; the minimum was 0, equivalent to a pristine pincer.

Results

How late in the intermolt can inhibition to the pincer claw be removed?

In most previous studies the snapper was removed a day or two after the shrimp molted, thus providing enough time for transformation and regeneration of claws that, by the next molt, both claws appeared at an advanced state. Can inhibition be removed later in the intermolt, even perhaps as late as the premolt stage? Snappers were autotomized in 37 shrimps at various stages of the molt cycle, as determined by measuring epidermal retraction and setal development in the pleopods (Aiken, 1973); pleopod stages may range from 0 (intermolt) to 5.5 (late premolt). Following ecdysis, the contralateral claws were examined and a transformation index, representing degree of transformation from pincer to snapper (Fig. 2), was calculated on a scale of 0 (the equivalent of a pristine pincer) to 10 (a pristine snapper). The results indicated that the effect of a snapper autotomy on the contralateral pincer was not restricted to the intermolt stage or even the early premolt stage. When done midway or even fairly late into the molt cycle—i.e., as late as pleopod stage 4.0—the pincer in some cases showed clear signs of transformation (Fig. 3). There was a gradation in the effect that was related to the time in premolt when the snapper was autotomized and the interval between the snapper autotomy and the ensuing ecdysis.

In addition, for 9 animals the transforming pincer was selected for histochemical analysis, to characterize the fiber type of the closer muscle. In these shrimp, the snapper had been autotomized at pleopod stages ranging from 0 to 3.5, and the transforming pincers had a transformation index ranging from 2.5 to 6.0. All 9 animals still retained the band of fast muscle that is unique to the pincer (Govind *et al.*, 1986), although in most animals it had begun to degenerate (Fig. 4). Muscle degeneration signifies that transformation had begun even in late premolt shrimp, suggesting that snapper-based inhibition is removed almost immediately and is unrelated to the stage of the molt cycle.

Is an intact transforming claw necessary to limit claw regeneration to a pincer at the contralateral snapper site?

We have shown previously that denervating the transforming pincer claw will allow regeneration of a pincer or a snapper claw at the contralateral snapper site; the appearance of a snapper claw implies the loss of snapper-based inhibition (Young *et al.*, 1994). To pursue this idea further, we wondered whether dactylotomy alone would

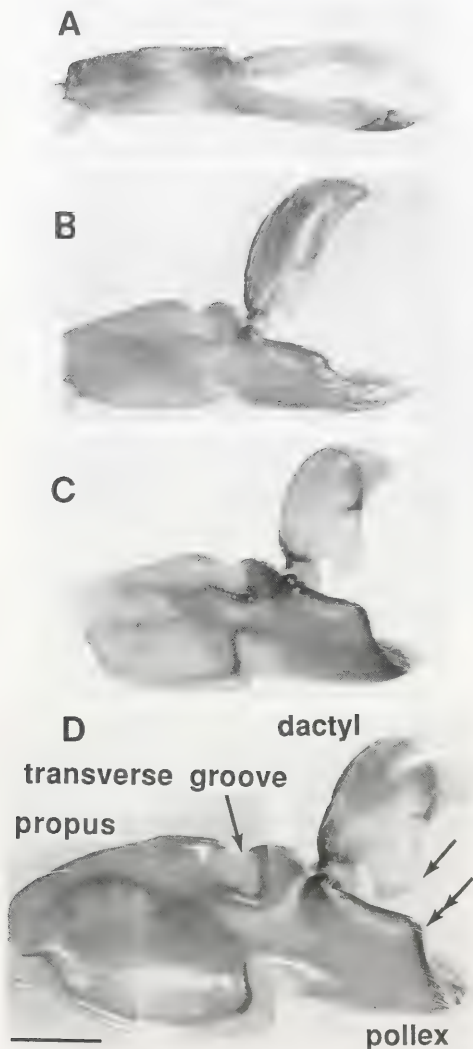


Figure 2. A pristine pincer claw (A) progressively developing via two selected stages (B, C) into a pristine snapper claw (D) characterized by a hammer (arrow) and socket (double arrow), a transverse groove, and hypertrophy of the entire claw. The slender pristine pincer claw (A) acquires all the snapper features after the first molt (B), and in subsequent molts (C, D) becomes hypertrophied with further accentuation of the snapper features. Scale bar 3 mm. $\times 6$

be equally effective. Ten animals were successfully manipulated so that the snapper was autotomized and the pincer dactylotomized. In all cases, the pincer transformed

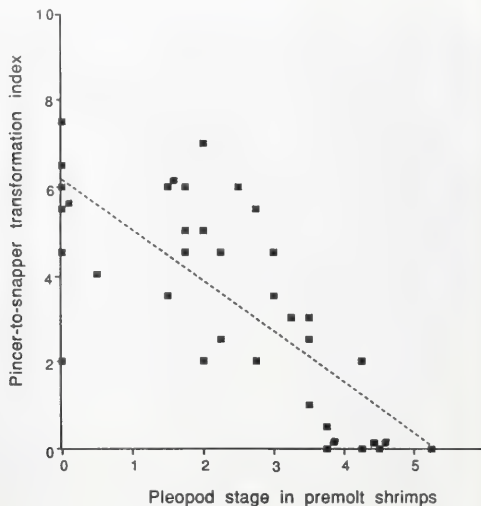


Figure 3. Relationship between pleopod stage in premolt shrimp subjected to snapper autotomy and pincer-to-snapper transformation index defined by 0 as pristine pincer and 10 as pristine snapper. The degree to which the pincer transforms is linearly related (regression line) to the stage at which the snapper is removed in premolt shrimps. $N = 37$, $r^2 = 0.577$ ($P < 0.005$), slope $b = -1.162$.

into a snapper; *i.e.*, it hypertrophied and developed a socket, despite lacking a dactyl (Table 1a). In 7 of those 10 shrimp, a snapper regenerated on the contralateral side, resulting in a symmetrical animal, albeit one snapper lacked a dactyl (Fig. 1C) and the regenerated snapper often did not grow to pristine proportions. In the other 3 shrimp, a pincer regenerated at the snapper site, resulting in a reversal of asymmetry. Thus pincer dactylotomy, mimicking a mild form of denervation, appeared to be sufficient to remove snapper-based inhibition of the regenerating claw.

Can snapper-based inhibition of a regenerating pincer claw be removed?

Loss of the pincer claw results in the regeneration of another pincer claw presumably because the contralateral snapper restricts regeneration at this site to a pincer (Wilson, 1903). Is it possible to remove this snapper-based inhibition by damaging the snapper when regeneration is taking place at the pincer site? We tested this possibility by removing the pincer claw and at the same time cutting the snapper dactyl. These procedures were successfully accomplished in nine shrimp (Table 1b). At the next molt, a normal-appearing pincer regenerated at the original pincer site in these shrimp. On the contralateral side the

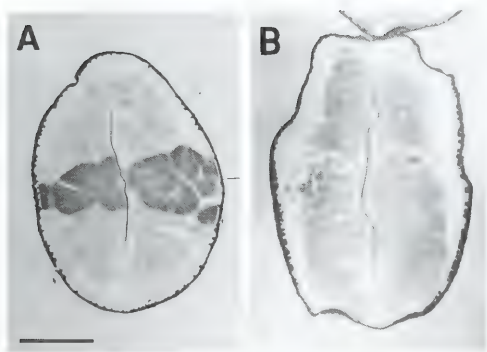


Figure 4. (A) Cross-section of a pristine pincer claw in which the closer muscle, stained histochemically for myofibrillar ATPase, shows a characteristic central band of fast fibers (dark-staining) flanked by slow fibers (light-staining). (B) Cross-section of a pincer claw transforming to a snapper in which fast fibers (dark staining) in the central band have degenerated, while the flanking slow fibers (light staining) are intact. Scale bar 1 mm. $\times 15$

intact snapper showed little regeneration of its cut dactyl for at least two subsequent molts. Snapper dactylotomy did not induce regeneration of a snapper claw at the pincer site.

In an earlier experiment, transecting nerve 2 in the transforming pincer claw permitted regeneration of a snapper claw at the old snapper site, implying the removal of snapper-based inhibition (Young *et al.*, 1994). Therefore, we transected nerve 2 from the autotomy plane to mid-merus in the pristine snapper claw and autotomized the pincer claw at the same time. The procedure was successful in 15 shrimp (Table Ic). None of these animals regenerated a snapper: 7 had regenerated a pincer by the end of the first molt cycle, 12 had a pincer by the end of the second molt cycle, and all had regenerated a pincer by the end of the third molt cycle. In 11 of these animals, snapper function was restored, but to varying degrees: 4 could open and close the dactyl but not snap, 5 could snap weakly, and 3 could snap with moderate force. In most cases, snapper function began returning near the end of the first molt cycle. Thus snapper denervation failed to bring about regeneration of another snapper claw at the pincer site.

Can snapper-side inhibition be weakened to permit regeneration of a snapper at the pincer site?

When both snapper and pincer claws are removed simultaneously, regeneration at these sites is of a similar type claw (Przibram, 1901), suggesting that snapper-based inhibition is present even in the absence of the snapper claw. Can this inhibition present on the snapper

side be weakened to allow regeneration of a second snapper on the contralateral side? We therefore tried removing both snapper and pincer claws at a time when snapper-based inhibition might not be well established. This would be the case immediately after reversal of asymmetry when the newly transformed snapper claw is not as elaborate nor as hypertrophied as a pristine snapper, nor is the newly regenerated pincer claw as well differentiated as the pristine pincer (Przibram, 1901; Wilson, 1903). Forty-seven animals were successfully snapper-autotomized and at the next molt, following a reversal of asymmetry, subjected to a paired autotomy. Relative to the most recent configuration, the location of the pincer and snapper was maintained in 33 and reversed in 14 animals (Table Id). Despite reversal of bilateral asymmetry, snapper-based inhibition remained intact.

We next tried snapper dactylotomy as a means of inducing pincer transformation. Out of 21 animals, 11 showed transformation of the pincer into a snapper while the damaged snapper repaired itself, resulting in shrimp with paired snapper claws. Following paired autotomy in these 11 animals, 8 regenerated limbs to mimic the original asymmetric configuration, but 3 regenerated double snappers (Table Ie). Both the regenerated snapper claws

Table I

Configuration of paired claws in snapping shrimp following regeneration at one or both sites in response to various manipulations

Manipulation	Claw configuration		#	%
	Snapper site	Pincer site		
a. Pincer dactylotomy with snapper autotomy	pincer snapper	snapper snapper	3	30
b. Pincer autotomy with snapper dactylotomy	snapper	pincer	9	100
c. Pincer autotomy with snapper denervation	snapper	pincer	15	100
d. Snapper autotomy at first molt; paired autotomy at second molt	snapper pincer	pincer snapper	33	70
	pincer snapper	snapper snapper	14	30
e. Snapper dactylotomy at first molt; paired autotomy at second molt	snapper snapper	pincer snapper	8	73
	snapper snapper	snapper snapper	3	27
f. Paired autotomy at first molt; paired autotomy at second molt	snapper pincer	pincer snapper	46	96
	pincer snapper	snapper snapper	1	2
g. Paired autotomy at first molt; paired autotomy at second molt and snapper-side limb bud immobilized at third molt	snapper snapper	pincer snapper	22	79
	snapper snapper	snapper snapper	6	21

were relatively small in comparison to the animal (Fig. 1D), although the limb on the original snapper side tended to be slightly larger than its counterpart. This was the first demonstration of a snapper claw regenerating at a pincer site with an extant snapper claw on the opposite side.

Can snapper-side inhibition survive successive claw loss?

A newly regenerated limb is usually smaller than a pristine limb, suggesting that the regenerating limb is in an immature state, which could probably be exaggerated with a second round of regeneration. Under these conditions, does snapper-side inhibition still prevail? A group of 48 shrimp were successfully subjected to two consecutive paired autotomies of their claws and the returning asymmetry observed (Fig. 5; Table 1f). After the paired claws had regenerated for the first time, 75% were asymmetric, although they were considerably smaller than the pristine claw; the remainder were no more advanced than stage 5 limb buds or stage 6 pincers (Govind and Read, 1994). After they had regenerated for the second time, they were even smaller. Only 34% showed slight asymmetry, and 66% resembled stage 5 limb buds or stage 6 pincers (Fig. 1B). Over the next two molts, however, these pincer-symmetric shrimp reverted to the original snapper/pincer configuration. Of 48 animals, 46 regenerated limbs to the original configuration, one experienced a reversal, and one regenerated paired snappers. These results show that snapper-based inhibition usually persisted through at least two successive paired autotomies, although occasionally it could be reversed or even absent.

As shown in the above experiment, after two successive paired autotomies the newly regenerated claws were small and the snapper was not highly differentiated. Occasionally only the faintest trace of a snapper—for example, a poorly developed hammer—was seen, and in overall dimension the claw was more like a pincer. To test the possibility that manipulation of the snapper-side limb, even in this relatively immature condition, could reverse claw asymmetry, we performed the following experiment. A group of 28 shrimp were subjected to two consecutive paired autotomies, then the snapper-side limb bud was glued shut, effectively restricting movement of the dactyl of this regenerating limb (Fig. 5; Table 1g). Most (22) of these animals regenerated claws resembling the original configuration. However, six animals regenerated paired snappers, showing that the limb bud at the pincer site was capable of developing into a snapper. When compared to the previous experiment in which regeneration was assessed after two successive paired autotomies, the number of snapper-symmetric shrimps in the present experiment proved to be significant ($X^2 = 5.612$, $P < 0.02$).

Can snapper-symmetry be maintained following claw loss?

The generation of adult shrimp with paired snapper claws by regeneration of a second snapper raises questions about the stability of this unusual condition. These snapper-symmetric shrimp retained their symmetry following a subsequent molt, at which time the second snapper assumed more pristine proportions (Fig. 1E). Symmetry was retained in five shrimp that underwent three subsequent molts, showing that once snapper-symmetry is established, it is retained through later molts. On the other hand, if the second snapper is removed, as was done in three shrimp after they had molted once, the shrimp regenerated a pincer in its place. Even if the second snapper was removed after three molts, as was done in two shrimp, a pincer regenerated in its place. Finally, in five shrimp with similar-sized paired snapper claws, removal of both claws resulted in the regeneration of paired asymmetric claws in the original configuration. Clearly, the snapper-symmetric condition is relatively stable but not permanent, as loss of one or both claws allows regeneration of asymmetric claws.

Discussion

Our experiments produced paired regenerated claws in the usual asymmetric configuration of pincer/snapper as well as, in a few cases, in the unusual symmetric configurations of pincer/pincer or snapper/snapper. The pincer-symmetric condition appears to be ephemeral, because in succeeding molts, given adequate time for development, one of the claws becomes a snapper. In contrast, the snapper-symmetric condition can be maintained over several molts, assuming a relatively permanent state. Indeed, we have maintained snapper-symmetric shrimp for five molts, providing there is no loss of or damage to the claws (Pearce and Govind, 1987). Loss of one or both claws in these snapper-symmetric shrimp immediately restores the asymmetric configuration of the paired claws upon regeneration. The observations that the pincer-symmetric condition is ephemeral and the snapper-symmetric condition is more stable tend to support the view that the pincer represents a stage in the development of the snapper, with the final condition of claw regeneration being that of a snapper (Wilson, 1903; Darby, 1934).

With this developmental sequence in mind, lateralization of the paired claws would be easily achieved if the snapper claw or its putative site arrested the development of the contralateral claw to a pincer state. Although the nature of the inhibitory mechanism is not known, considerable evidence suggests that it has a neural basis and that inhibition is removed most readily with loss of the entire claw but also with nerve transection (Mellon and Stephens, 1978), closer muscle tenotomy (Govind *et al.*, 1988), or dactylotomy (Read and Govind, 1997); in other

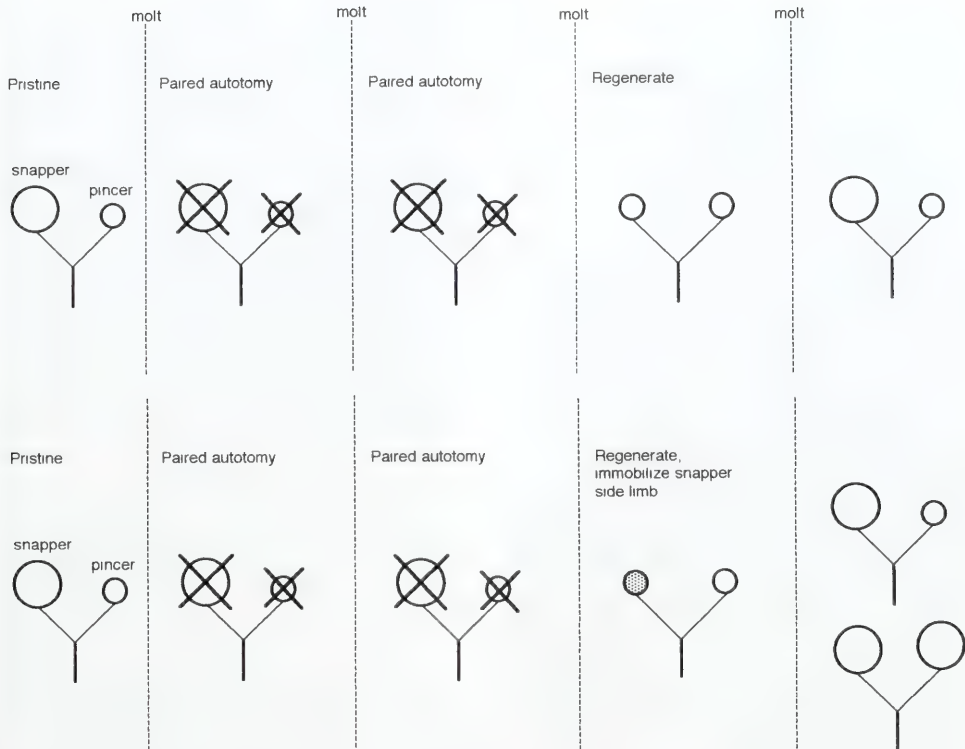


Figure 5. Pictorial representation of the paired asymmetric snapper (large circle) and pincer (small circle) claws of adult shrimp in two experiments. In the upper series the shrimps underwent two successive paired autotomies and regenerated paired pincerlike claws after the third molt; after the fourth molt, these claws differentiated into snapper/pincer claws in the pristine configuration. The same experiment was repeated in the lower series, but after the third molt the snapper-side limb was immobilized (shaded); at the fourth molt, paired claws appeared in the pristine snapper/pincer configuration but also in a snapper/snapper configuration.

words, with some damage to the nervous system. Once the inhibition is removed the pincer continues its development to a snapper, even as late in the intermolt as the premolt stage, when the new exoskeleton is being laid down. These changes involve the exoskeleton and closer muscle composition, whereas the motor innervation (Stephens and Mellon, 1979; Quigley and Mellon, 1984; Mellon *et al.*, 1981), sensory innervation (Govind and Pearce, 1988), and vascularization (Guchardi and Govind, 1990) occur later.

The next step in the lateralizing mechanism is for the claw advancing from the pincer to the snapper stage to exert an inhibitory influence on the regenerating contralateral claw and hold its development to the pincer stage.

Elimination of the inhibitory influence should allow the regenerating claw to develop into a snapper, and this was the case in shrimp in which removing the closer muscle in the transforming claw or transecting its nerve 2 allowed regeneration of a snapper claw on the opposite side (Young *et al.*, 1994). We now report that simply cutting the dactyl of the transforming claw is sufficient to eliminate its inhibitory influence and permit regeneration of a snapper claw, resulting in shrimp with paired snapper claws. The first snapper arises because the pincer, released from its inhibition by snapper autotomy, continues its development to a snapper. The second snapper arises because, owing to dactyloctomy of the transforming snapper, the newly regenerating claw is not restricted to a pincer stage.

Under this scheme the pincer would also advance to the snapper stage if the snapper-based inhibition was removed without loss of the snapper claw. We know that this happens readily when neural input is reduced in the snapper claw and the existing pincer continues its development to a snapper, resulting in shrimp with paired snapper claws (Mellon and Stephens, 1978; Govind *et al.*, 1988). Along similar lines is our earlier finding that cutting nerve 2 of the transforming claw (Young *et al.*, 1994) or our present finding that cutting off the dactyl of the transforming claw allows the regeneration of another snapper claw on the opposite side. But when these same surgeries were performed on a pristine snapper claw and the pincer claw was autotomized at the same time, another pincer regenerated in its place. In this case the presence of a pristine, although damaged, snapper was sufficient to inhibit regeneration to a pincer stage, but the presence of a damaged transforming snapper was not. Bearing in mind that the snapper claw has almost twice as many axons as its pincer counterpart in the pristine condition (Govind and Pearce, 1988), it is likely that dactylotomy of a transforming claw results in a much greater reduction of axons compared to the opposite side than does dactylotomy of the pristine snapper claw. Moreover, because dactylotomy affects similar structures in the transforming or pristine snapper claw, the results cast doubt on the possibility that qualitative aspects of the innervation are responsible for the different outcomes and point more to quantitative aspects. Limb regeneration in amphibians is dependent on a minimal amount of nerve in the blastema irrespective of the qualitative composition of the nerve, whether sensory or motor (Singer, 1978).

Quantitative differences in innervation between the two sides in adult snapping shrimp may also help explain the results of our experiments with paired autotomies; whether the paired autotomies were performed following reversal of asymmetry or in quick succession, the paired claws regenerated in an asymmetric configuration. The greater neural innervation to the snapper side would serve to inhibit the contralateral side even in the absence of the claws. The paired autotomy experiments suggest that claw lateralization in adult snapping shrimp has a central locus, similar to that in juvenile lobsters where differential reflex activity from the paired claws lateralizes the ganglion into major and minor sides during a critical developmental period (Govind and Pearce, 1986). Once laterality is established in juvenile lobsters it remains fixed for the entire life of the animal, and claw loss results in the regeneration of a similar claw type. Conversely, in snapping shrimp claw laterality is not fixed and can be constantly reversed. The present experiments reveal a very strong intrinsic lateralizing mechanism in the form of snapper-based inhibition that, because it operates in the absence of the claws, must reside centrally. The direction of the laterality can be readily changed by means of input from the claws in

the form of the removal of the snapper-based inhibition of the contralateral pincer claw. A useful analogy for the lateralizing mechanism is that of a seesaw in which the beam, balanced on its fulcrum, can assume one of two inclined positions but rarely a horizontal position.

Our assumption that the snapper-based inhibition of the pincer claw is neural in origin gained some support from our finding that restricting dactyl movements of the regenerating snapper claw permitted the regenerating claw on the opposite side to develop as a snapper. In these few cases, interfering with reflex activity of the regenerating snapper claw was sufficient to remove inhibition of the pincer claw. Differences in reflex activity between the two sides were also responsible for determining claw bilateral asymmetry in developing lobsters (Govind and Pearce, 1986). In snapping shrimp, differences in reflex activity are subserved by differences in numbers of axons to the paired claws (Govind and Pearce, 1988).

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H O T P A P E R S

**SIGNAL TRANSDUCTION/
CELL ADHESION BIOLOGY**

N. Funayama, F. Fagotto, P. McCrear, B.M. Gumbiner, et al.
"Embryonic axis induction by the armadillo domain of β -catenin: Evidence for intracellular signaling." *Journal of Cell Biology*, 128:95 (Cited in more than 60 publications as of February 1997)

Comments by Barry Gumbiner, Memorial Sloan-Kettering Cancer Center

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